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**(54) Title:** CHITINASE, DNA CODING THEREFOR AND PLANTS CONTAINING SAME

### **(57) Abstract**

The present invention provides a novel class of plant chitinases, which have endo-chitinase activity, antifungal activity and a molecular weight of about 40 to 43 kDa as judged by Sodium Dodecyl Sulphate Polyacrylamide electrophoreses. The invention further provides antifungal compositions comprising as active ingredient a chitinase according to the invention. According to a different aspect of the invention an isolated DNA sequence is provided which comprises an open reading frame encoding a novel chitinase. The invention also encompasses a plant expressible recombinant DNA sequence which comprises a DNA sequence encoding a chitinase according to the invention, as well as plant cells and plants which have such recombinant DNA incorporated into their genome.

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## CHITINASE, DNA CODING THEREFOR AND PLANTS CONTAINING SAME

FIELD OF THE INVENTION

The present invention relates to chitinases from plant origin, DNA coding therefor, and plants which have been transformed to contain and express said recombinant polynucleotides to produce or overproduce said chitinase. The invention further provides methods to protect plants from pathogen attack, as well as plants obtainable thereby. The invention further comprises antipathogenic compositions containing as active 10 ingredient a chitinase according to the invention.

STATE OF THE ART

Plants react to stresses such as attack by pathogens, chemical treatment and wounding, by accumulating a large number of proteins. One 15 of the best studied plant responses is the induced expression of so-called pathogenesis-related proteins, PR-proteins, (Bowles, D. 1990, Annu. Rev. Biochem. 59, 873-907); Linthorst, H.J.M., 1991, Crit. Rev. Plant Sci. 10, 123-150).

PR-proteins can be classified into at least five families (PR-1 to 20 PR-5) based on structure and/or activity. In tobacco, within each family of PR-proteins both intracellular (class-I) and extracellular (Class-II) isoforms are distinguished.

Among the PR-proteins are chitinases, which constitute the PR-3 family (Legrand M., et al, 1987, Proc. Natl. Acad. Sci. USA, 84, 25 6750-6754). The class-I chitinases possess antifungal activity (Broekaert W.F. et al, 1988, Physiol. Mol. Plant Pathol. 33, 319-331), especially in combination with  $\beta$ -1,3-glucanase (Schlumbaum A. et al, 1986, Nature 324, 365-367), classified as the PR-2 family (Kauffmann S. et al, 1987, EMBO J. 6, 3209-3212).

30 The occurrence of chitinases and the cloning of their corresponding cDNAs has been described for a large number of plant species, both dicotyledonous and monocotyledonous species (for a recent review, vide Collinge D.B. et al, 1993, The Plant Journal 3(1), 31-40). Chitinases are classified according to their primary structure into three classes 35 (Shinshi H. et al, 1990, Plant Mol. Biol. 14, 357-368). Recently chitinases have been found that do not fit into this classification: these chitinases have been proposed to form class-IV. The class I

chitinases are characterised by the presence of an approximately 40 amino acid cysteine rich domain.

The class-II chitinases resemble the class-I chitinases, *inter alia* by their amino acid sequence homology, but the former lack a cystein-rich 5 domain.

The class-III chitinases do not share sequence similarity with chitinases of either of the classes I or II. The class-IV chitinases, as proposed by Collinge et al. *supra*, comprise sugar beet chitinase IV (Mikkelsen J.D. et al, 1992, In Advances in Chitin and Chitosan (Brine, 10 C.J. Sandford, P.A. and Zikakis, J.P. eds. Amsterdam Elsevier, in press) and the basic rape chitinase ChB4 (Rasmussen U. et al, 1992, Planta, 187, 328-334), as well as the acidic bean PR4 chitinase (Margis-Pinheiro M. et al, 1991, Plant. Mol. Biol. 17, 247-253), show sequence similarity with the class I chitinases, contain a N-terminal cystein-rich domain, but are 15 smaller than the class-I chitinases due to a number of deletions. The molecular weights of plant chitinases range on average from about 26 to about 36 kDa.

By cloning the genes coding for PR-proteins, altering their expression patterns by placing the genes under the control of 20 non-natural, e.g. constitutive, regulatory elements, and reintroducing those constructs in plants, it is possible to reduce the susceptibility of plants to pathogen-attack, in particular fungal attack (European patent application 0 440 304 A1; EP 0 460 753). Simultaneous expression in transgenic plants of class-I chitinases and B-1,3-glucanases produces 25 a marked enhancement of fungal resistance in transgenic plants, showing the value of combining antipathogenic proteins.

There is a continuous need for the identification of new chitinases for use in antifungal compositions and/or for the cloning of cDNAs or 30 genes encoding such chitinases, to study the effect of expressing these cDNAs or genes in transgenic plants and evaluate the susceptibility of said plants to pathogenic attack, in particular to fungal attack.

It is an object of the present invention to provide novel antipathogenic proteins, DNA coding therefor, as well as plants which contain and express such DNA, to impart on such plants a reduced 35 susceptibility to pathogen attack, in particular fungal attack.

SUMMARY OF THE INVENTION

The present invention provides a protein free from other plant proteins which has endo-chitinase activity, antifungal activity and a molecular weight of about 40 to 43 kDa as judged by Sodium Dodecyl Sulphate Polyacrylamide electrophoresis. A more preferred protein is one which is obtainable from leaves of TMV-induced tobacco plants. A still more preferred protein is one having an amino acid sequence as represented in SEQIDNO: 2 or 8, or a fragment thereof having chitinase activity. Also provided are proteins according to the invention wherein 10 one or more amino acids are substituted, deleted, or added without abolishing chitinase activity.

Another embodiment of the invention is an antifungal composition comprising as active ingredient a protein according to the invention. A preferred composition is one which comprises in addition to said protein 15 a further protein acting synergistically with the said protein on fungi. Further preferred compositions comprise a  $\beta$ -1,3-glucanase, preferably a basic  $\beta$ -1,3,-glucanase and/or a chitinase, preferably a class I chitinase, more preferably from tobacco.

A further embodiment of the invention is an isolated DNA sequence 20 which comprises an open reading frame encoding a protein according to the invention, preferably one having the sequence as represented in SEQIDNO: 2 or SEQIDNO: 8, or DNA sequences hybridising therewith under stringent conditions.

The invention also comprises a plant expressible recombinant DNA sequence which comprises a DNA sequence encoding a protein according to 25 the invention, or a precursor of said protein, said DNA sequence being physically linked to regulatory elements necessary for expression of said DNA sequence in plants to produce said protein. A preferred plant expressible recombinant DNA sequence is one, wherein said DNA sequence 30 encodes a protein precursor having the amino acid sequence represented by SEQIDNO: 2 or SEQIDNO: 7. Another preferred plant expressible recombinant DNA sequence comprises the DNA sequence as represented in SEQIDNO: 1 or 7.

The invention also encompasses a cloning vehicle or a vector 35 containing a DNA sequence according to the invention, as well as bacterial strains containing such a cloning vehicle or vector. Also provided are bacterial strains of the genus *Agrobacterium* containing a

vector suitable for transferring DNA according to the invention to plant cells and plants.

Other embodiments of the invention comprise plant genomes, plant cells comprising such genomes and plants regenerated from such plant 5 cells, which as a result thereof produce or overproduce proteins according to the invention. Ideally, expression of DNA encoding proteins according to the invention is optimised to render plants less susceptible to pathogen attack, in particular fungal attack.

The invention further comprises plant material such as a bulb, 10 flower, fruit, leaf, pollen, root or root culture, seed, stalk, tubers or microtubers, and the like, comprising one or more cells which have incorporated therein plant expressible DNA according to the invention.

The invention also provides a method for breeding a plant variety which has reduced susceptibility to fungi, characterized in that at least 15 one of the parental lines has a recombinant DNA genome according to the invention, as well as a method for reducing the damage to plants under cultivating conditions. Further aspects of the invention, various ways to practice the invention and some advantages are outlined in the detailed description.

20 The invention is illustrated by the following Figures.

#### DESCRIPTION OF THE FIGURES

Fig. 1. Nucleotide sequence of the Cluster-A gene and a homologous cDNA clone. The complete nucleotide and amino acid sequence of the 25 genomic cluster-A clone are shown in line b and line c, respectively. Above the nucleotide sequence only the nucleotides which are different in the cDNA clone are indicated in line a. Similarly, below the Cluster-A protein sequence only the amino acids which are different in the cDNA sequence are indicated in line d. The amino acid sequence determined by 30 sequencing the protein are underlined. The putative cleavage site of the signal peptide, between residues (Ser (S) and Gln (Q), is indicated by an arrow.

Fig. 2. Southern blot analysis of three clones containing sequences 35 homologous to the tobacco Cluster-A gene. Three genomic clones of tobacco (clone-56; 59; 66) were purified and digested with either BamHI, HindIII, PstI, SstI or XbaI, lanes 1 to 5 respectively, and electrophoresed on an

0.8% agarose gel. The gel was blotted and hybridized with the cluster-A cDNA and washed at 65°C in 0.1 x SSPE, 0.1% SDS.

Fig. 3. Northern blot analysis of the tobacco Cluster-A mRNAs upon 5 different forms of stress. Equal amounts of total RNA isolated from non-stressed healthy tobacco leaves (H) or from leaves infected with TMV (T), sprayed with eptephon (E), wounded by cutting (W) or irradiated with UV-light (U) were hybridized to a Cluster-A cDNA probe.

10 Fig. 4. Alignment of Cluster-A of *Nicotiana tabacum* with exo-chitinases from *Bacillus circulans*, *Serratia marcescens*, *Streptomyces plicatus*, *Brugia malayi* (nematode) and *Mus musculus* (mouse). The black boxes indicate regions with a high degree of identity according to the multiple alignment analysis by Schuler G.D. et al, 1991, Protein Struct. 15 Funct. Genet. 2, 180-190).

Fig. 5. Over-production of Cluster-A in *E. coli*. (A): On a 12.5% SDS-polyacrylamide gel different samples, total fraction (T), cell debris (D) and soluble fraction (S), were analyzed. (B): Immunoblot analysis of 20 an identical protein gel with specific detection of Cluster-A proteins.

Fig. 6. Purification of the Cluster-A protein from TMV-infected tobacco leaves. SDS-polyacrylamide gel shows the protein profile of different purified fractions. A: sample of desalted crude leaf-extract, 25 B: protein pool after S-sepharose cation-exchange chromatograph, C: protein pool after Chelating Superose column, D: purified Cluster-A after gel-filtration chromatography. The lane "Mr" shows pre-stained markers with molecular weights as indicated.

30 Fig. 7. Cellular localization of Cluster-A. The antiserum to the Cluster-A protein was used to determine the cellular localization of tobacco Cluster-A. Healthy non-infected and TMV-infected tobacco plants were analysed. Samples of a total protein fraction (lane a), leaf fraction without extracellular washing fluid (lane b), and EF-fraction 35 (lane c), were separated on a 12.5% SDS-gel and electroblotted to a PVDF membrane, and used for detection of Cluster-A proteins.

Fig. 8. Growth inhibition of *Fusarium solani* by the synergistic activity of Cluster-A and class I  $\beta$ -1,3-glucanase proteins. The effect of Cluster-A on fungal growth *in vitro* was tested by the addition of Cluster-A to pre-germinated spores of *Fusarium solani*, either alone, or 5 in combination with class I  $\beta$ -1,3-glucanases. Amounts of protein (in  $\mu$ g) per well are indicated. After three days of incubation at 20°C the fungal mycelia were visualised by staining with lactophenol cotton blue.

Fig. 9. Schematic representation of vector pMOG183, a derivative of 10 pMOG181 wherein the EcoRI recognition site is replaced by a SstI site this vector is freely available from MOGEN International N.V. upon request.

Fig. 10. Schematic representation of vector pMOG185, a derivative of 15 pMOG183 wherein the EcoRI recognition site changed into a XbaI recognition site.

Fig. 11. pMOG800: A suitable binary vector for Agrobacterium-mediated plant transformation, containing a plant expressible kanamycin resistance 20 gene for selection of transformants and a multiple cloning site for insertion of expression cassettes.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel class of chitinases which 25 can be isolated from plants. The invention provides in particular an approximately 40 - 43 kDa protein from TMV-induced tobacco leaves. This protein was found to be encoded by an open reading frame which closely resembles a cDNA previously called cluster-A (Hooft van Huijsduijnen et al (Hooft van Huijsduijnen R.A.M. et al, The EMBO Journal 5(9), 2057-2061). In this reference 6 clusters (A to F) of mRNAs have been described which are TMV- and salicylate-inducible in tobacco. The authors characterized the mRNAs of the six clusters by hybrid-selected *in vitro* translation. The translation products of the hybrid-selected mRNA corresponding to the largest cluster-A cDNA clone produced a complex 35 pattern of proteins of 40, 35, 31, 28 and 25 kDa respectively (See lane 6, Fig. 3 of Hooft van Huijsduijnen et al, supra). The protein actually produced by this cDNA sequence was never determined. None of the protein

fragments was ever purified, nor was the structure of either of these fragments determined. No physiological activity was known for any of these protein fragments either.

According to the present invention the protein corresponding with 5 this Cluster-A cDNA was purified to homogeneity from TMV-induced tobacco leaves using an antiserum. The antiserum was raised against a purified protein produced in *E. coli* transformed with a cDNA clone from tobacco hybridising with PROB40. The clone PROB40 was known to cross-hybridise with Cluster-A cDNA, (Hooft van Huijsduinen et al, (supra)).

10 Using this antiserum in the identification and purification process, Cluster-A protein could be isolated from TMV-infected tobacco leaves in sufficient quantity to study its physiological and antipathogenic activity. A partial amino acid sequence of the isolated protein was obtained from sequencing the purified protein from tobacco; 15 it was almost identical with the amino acid sequence deduced from the cDNA (Fig. 1).

The Cluster-A protein was found to possess low chitinase activity on tritiated chitin as a substrate, about 250-500 times lower than the class I chitinases from tobacco. However, when tested with a colorimetric 20 assay on a soluble chitin substrate, its activity was found to be about two fold higher than the class I chitinases. No exochitinase activity or lysozyme activity could be detected. Altogether, it was concluded that the Cluster-A represented an endochitinase, but with different properties as those belonging to class I endochitinases. It was concluded that 25 Cluster-A is the first member of an entirely new class of chitinases in plants. The cluster-A protein was found to be predominantly located intracellularly.

The Cluster-A protein appeared to possess antifungal activity in an 30 *in vitro* assay against the fungus *Trichoderma viride*. In combination with  $\beta$ -1,3-glucanases this activity was synergistically enhanced as judged from the sensitivity of a number of test fungi. Both lysis and growth inhibition could be detected on *Fusarium solani* and *Alternaria radicina* when Cluster-A protein and  $\beta$ -1,3-glucanase were tested in combination.

The cDNA obtained after screening of the cDNA library with PROB40 35 was used as a probe to screen a genomic library to isolate the gene encoding the Cluster-A protein. One hybridising clone was characterised and its nucleotide sequence determined. The complete sequence of the

cluster-A gene, as well as part of the promoter region, is depicted in Fig. 1. (SEQIDNO: 7). Analysis of the deduced amino acid sequence shows that the Cluster-A protein is probably produced as a larger precursor of which a signal peptide (about 25 amino acids) is cleaved off: the 5 putative cleavage site is indicated by an arrow. Southern analysis (Fig. 2) revealed that the Cluster-A gene is part of a small gene family of about 4 genes.

This novel chitinase, hereinafter referred to as Cluster-A protein, may suitably be used as the active ingredient in antipathogenic, 10 particularly antifungal, compositions, preferably in combination with other proteins with which it works synergistically, such as  $\beta$ -1,3-glucanases. Suitable concentration ranges of Cluster-A protein are from about 0.01  $\mu\text{g}/\text{ml}$  to about 100  $\mu\text{g}/\text{ml}$ , preferably, from about 0.1  $\mu\text{g}/\text{ml}$  to about 10  $\mu\text{g}/\text{ml}$ . Antifungal compositions according to the 15 invention may be used in agriculture to combat phytopathogenic fungi in a broad sense; examples are uses in horticulture, arboriculture, ornamental gardening, home gardening, flower culture, and the like. Similarly the antifungal effect of Cluster-A protein may be useful in other industrial areas such as in preservatives for beverages, foods, cosmetics, and the 20 like.

Furthermore, DNA coding for Cluster-A protein, either a cDNA or a genomic sequence, may be cloned behind the regulatory sequences required for expression of the DNA in plants, in order to produce or overproduce Cluster-A protein in plants transformed with the said DNA. The necessary 25 regulatory elements comprise at least a transcriptional initiation region and a translational initiation region functional in plants. Regulatory sequences may include additional elements such as enhancers to promote transcription. Enhancers may increase expression in a constitutive fashion or in a tissue-specific or developmentally, or environmentally 30 regulated fashion.

A preferred mode of expression according to the invention is an essentially relatively constitutive mode of expression, preferably at high levels (for purposes of this invention about 0.1% of soluble protein in leaves is regarded as a relatively high expression level). With a 35 relatively constitutive expression pattern is meant a pattern that is not drastically regulated according to the developmental stage of the plant and which is not tissue-specific. A promoter which is generally regarded

by those of skill in the art to be relatively constitutive and not drastically developmentally regulated is the CaMV 35S promoter. A preferred version of such promoter contains a so-called double enhancer. Other relatively constitutive promoters which are suitable to be used in conjunction with the present invention may be obtained from plant viruses (e.g. CaMV 19S promoter, Figwort Mosaic Virus promoter, and the like), as well as from plant genes. Relatively constitutive promoters are also derivable from the T-DNA region of Ti- and Ri-plasmids from *Agrobacterium*, either or not flanked by transcriptional enhancer sequences. From the literature it is known that the sequence between -343 and -90 of the CaMV 35S promoter increases the activity of the CaMV 35S promoter (Kay R. et al (1987), *Science* 236, 1299-1302) and presumably also of other constitutive promoters (e.g. mannopine synthase promoter: US Patent 5,106,739).

15 Examples of light-inducible high-level promoters are the ribulose bisphosphate carboxylase small subunit (rbcSSU) promoter and the chlorophyl a/b binding protein (Cab) promoter, both of which may be used in conjunction with the present invention.

It may be desirable to restrict expression of the introduced chimeric genes to one or a few pre-selected organs or tissues, for instance those that are targets for fungal attack, such as roots, epidermal cells, and the like. A well known example of a tissue-specific promoter is for example the patatin class-II promoter. Also root-specific promoters may be used. Suitable promoters for use in a process according to the invention may also be wound-inducible.

The invention also embraces the use of hybrid promoters, i.e. promoters that comprise elements derived from regulatory elements of different genes.

Plant expressible genes generally comprise a so-called terminator sequence including a polyadenylation signal, it is also possible to use the terminator of the gene according to the invention, this is not critical.

The word 'gene' as used here is meant to comprise cDNAs as well as transcribed region of a genomic sequence, either of which may be synthetic or partially synthetic. If desired codon usage may be changed for use in e.g. monocotyledonous plant hosts, or non-plant hosts. 'Plant expressible gene' shall mean a DNA sequence which is operably linked to

regulatory sequences required for transcription in a plant cell and which yields RNA upon transcription which, after processing, can be translated into protein. A gene is 'plant expressible' if it is expressed at least in one tissue in one particular phase of the life cycle of the plant. A 5 gene is understood to be plant expressible even if it is not expressed at all stages during development or only after induction by certain internal or external stimuli.

A plant expressible recombinant DNA sequence according to the invention shall mean to comprise any DNA sequence which at least combines 10 two sequences that are not associated naturally. For instance plant expressible recombinant DNA may comprise genes which comprise combinations of functional regions of a eukaryotic gene such as enhancers, transcription/translation initiation regions, coding regions, non-translated leaders, signal sequences, vacuolar targeting sequences, 15 terminator sequences, introns, exons, and the like, or parts thereof. Any deletion, addition, or substitution of one of those elements yielding an expression level or pattern different from the natural situation is envisaged by the present invention. A modification does not necessarily have to increase expression levels. The present invention for instance 20 clearly envisages the truncation of the C-terminal part of the protein according to the invention in order to redirect its accumulation to the apoplastic (extracellular) space, which may be a preferred location for an antipathogenic protein. Modifications as this truncation may be the only modification of the gene, leaving the remainder of the open reading 25 frame, as the other sequences determining its functioning (such the promoter, terminator, introns and exons, and the like) untouched, yet bring the DNA encoding a protein according to the invention under the definition of a plant expressible recombinant DNA.

An effective site of action of antifungal proteins in the 30 protection of transformed plants against plant pathogenic fungi is believed to be the apoplastic space. Hence, plants may be transformed with a recombinant DNA construct encoding a plant expressible gene according to the invention which exerts its action in the apoplastic space of the plant, either naturally or by virtue of genetic 35 modification.

Genes encoding proteins that naturally occur in the plant cell vacuole are modified such that the C-terminal amino acids involved in

vacuolar targeting are not present in the translation product (e.g. by introducing a translational stopcodon in the C-terminal end of the coding region of the gene, or otherwise). The effect of this truncation is targeting of the vacuolar protein to the apoplastic space (for details of 5 the procedure see EP 440 304 A1).

Each of the above mentioned manipulations are well known to those of skill in the art and the effects thereof on expression level, targeting and disease resistance can readily be evaluated using standard techniques.

10 Generally, transformed plants are evaluated for the presence of the desired properties and/or the extent to which the desired properties are expressed. A first evaluation may include the level of expression of the newly introduced genes, the level of fungal resistance of the transformed plants, stable heritability of the desired properties, field trials and 15 the like.

Secondly, if desirable, the transformed plants can be cross-bred with other varieties, for instance varieties of higher commercial value or varieties in which other desired characteristics have already been introduced, or used for the creation of hybrid seeds, or be subject to 20 another round of transformation and the like.

The combination of Cluster-A protein with tobacco  $\beta$ -1,3-glucanase produces a synergistic antifungal effect *in vitro*. Similarly, a synergistic antifungal effect is expected when both proteins are produced or overproduced *in planta*. This is deduced from the fact that when 25 combinations of chitinases and glucanases produce a synergistic effect *in vitro* they do so *in vivo* as well (EP 440 304 A1).

Examples of proteins that may be used in combination with antifungal proteins according to the invention include, but are not limited to,  $\beta$ -1,3-glucanases and other chitinases such as those 30 obtainable from barley (Swegle M. et al, 1989, Plant Mol. Biol. 12, 403-412; Balance G.M. et al, 1976, Can. J. Plant Sci. 56, 459-466 ; Hoj P.B. et al, 1988, FEBS Lett. 230, 67-71; Hoj P.B. et al, 1989, Plant Mol. Biol. 13, 31-42 1989), bean (Boller T. et al, 1983, Planta 157, 22-31; Broglie K.E. et al. 1986, Proc. Natl. Acad. Sci. USA 83, 6820-6824; 35 V\_geli U. et al, 1988 Planta 174, 364-372); Mauch F. & Staehelin L.A., 1989, Plant-Cell 1, 447-457); cucumber (Métraux J.P. & Boller T. (1986), Physiol. Mol. Plant Pathol. 28, 161-169); leek (Spanu P. et al, 1989,

Planta 177, 447-455); maize (Nasser W. et al, 1988, Plant Mol. Biol. 11, 529-538), oat (Fink W. et al, 1988, Plant Physiol. 88, 270-275), pea (Mauch F. et al 1984, Plant Physiol. 76, 607-611; Mauch F. et al, 1988, Plant Physiol. 87, 325-333), poplar (Parsons, T.J. et al, 1989, P.N.A.S. 86, 7895-7899), potato (Gaynor J.J. 1988, Nucl. Acids Res. 16, 5210; Kombrink E. et al 1988, Proc. Natl. Acad. Sci. USA 85, 782-786; Laflamme D. and Roxby R., 1989, Plant Mol. Biol. 13, 249-250), tobacco (e.g. Legrand M. et al 1987, Proc. Natl. Acad. Sci. USA 84, 6750-6754; Shinshi H. et al. 1987, Proc. Natl. Acad. Sci. USA 84, 89-93), tomato (Joosten 10 M.H.A. & De Wit P.J.G.M. 1989, Plant Physiol. 89, 945-951), wheat (Molano J. et al, 1979, J. Biol. Chem. 254, 4901-4907), and the like.

The cloning of plant genes corresponding to proteins that can suitably be used in combination with genes encoding antifungal proteins according to the invention and the over-expression of such genes in 15 transgenic plants, as well as the manipulation of expression (including targeting) and the assessment of disease resistance *in planta* is all within the scope of those of skill in the art, as is exemplified *inter alia* in Application European patent application 0 440 304 A1, and European patent application 0 460 753 A2. Both applications are herein 20 incorporated by reference.

Transgenic plants which harbour more than one chimeric antifungal gene may be obtained by a variety of processes including the following:

- A. the use of one recombinant polynucleotide, e.g. a plasmid, with a number of modified genes physically coupled to one selection marker gene.
- 25 B. Cross-pollination of transgenic plants which are already capable of expressing one or more chimeric genes coupled to a gene encoding a selection marker, with pollen from a transgenic plant which contains one or more gene constructions coupled to another selection marker. Afterwards the seed, which is obtained by this crossing, is selected on 30 the basis of the presence of the two markers. The plants obtained from the selected seeds can afterwards be used for further crossing.
- C. The use of a number of various recombinant polynucleotides, e.g. plasmids, each having one or more chimeric genes and one other selection marker. If the frequency of co-transformation is high, then selection on 35 the basis of only one marker is sufficient. In other cases, the selection on the basis of more than one marker is preferred.
- D. Consecutive transformations of transgenic plants with new, additional

chimeric genes and selection marker genes.

E. Combinations of the above mentioned strategies.

The actual strategy is not critical with respect to the described invention.

5 Any plant species that is subject to some form of fungal attack may be provided with one or more plant expressible gene constructs according to the invention. Likewise any plant subject to some form of fungal attack may be treated with a composition comprising an antifungal protein according to the invention.

10 Hence, "plants" for the purpose of this invention shall mean gymnosperms as well as angiosperms, dicotyledonous plants as well as monocotyledonous plants, be they used in agriculture, horticulture, forestry, (indoor) gardening, or any other form of activity involving plants, either for direct use as food or feed, or for further processing  
15 in any kind of industry, for extraction of substances, for decorative purposes, propagation, cross-breeding, or any other use.

In principle any transformation method may be used to introduce a plant expressible gene according to the invention into a plant species of choice. Generally, useful methods are the calcium/polyethylene glycol  
20 method for protoplasts (Krens, F.A. et al, 1982, *Nature* 296, 72-74; Negrutiu I. et al, June 1987, *Plant Mol. Biol.* 8, 363-373), electroporation of protoplasts (Shillito R.D. et al, 1985 *Bio/Technol.* 3, 1099-1102), microinjection into plant material (Crossway A. et al, 1986, *Mol. Gen. Genet.* 202, 179-185), (DNA or RNA-coated) particle bombardment  
25 of various plant material (Klein T.M. et al, 1987, *Nature* 327, 70), infection with viruses and the like.

In a preferred embodiment of the invention use is made of *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP-A 120 516 and  
30 U.S. Patent 4,940,838).

Generally, after transformation plant cells or explants are selected for the presence of one or more markers which are encoded by plant expressible genes co-transferred with the plant expressible gene according to the invention, whereafter the transformed material is  
35 regenerated into a whole plant.

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation

and fertile transgenic plants can be regenerated from transformed cells or protoplasts. Presently, preferred methods for transformation of monocots are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, et al, 1989, *Nature* 338, 274-276). Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus* bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, *Plant Cell*, 2, 10 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, *Plant Mol. Biol.* 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the 15 embryogenic suspension cultures (Vasil, 1990 *Bio/Technol.* 8, 429-434). The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially important crops such as corn and rice are readily amenable to transformation by 20 *Agrobacterium* strains (Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) *Plant. Physiol.* 95, 426-434) Hiei Y et al.(1994) *Plant Journal* 6, in press.

Plants and plant parts producing or over-producing a novel chitinase according to the invention, either alone or in combination with 25 other antifungal genes, or genes encoding proteins which work synergistically with the chitinase according to the invention, may be used to evaluate pathogen resistance, in particular fungal resistance. Subsequently, the more resistant lines may be used in breeding programs to yield commercial varieties with enhanced pathogen, in particular 30 fungal resistance.

Plants with reduced susceptibility against pathogen or fungal attack, may be used in the field or in greenhouses, and subsequently be used for animal feed, (direct) consumption by humans, for prolonged storage, used in food- or other industrial processing, and the like. The 35 advantages of the plants, or parts thereof, according to the invention are a lesser need for fungicide treatment, lowering costs of material, labour, and environmental pollution, or prolonged shelf-life of products

(e.g. fruit, seed, and the like) of such plants.

Furthermore, post-harvest losses may be reduced due to the presence of enhanced levels of the chitinase according to the invention as well.

Further advantages of the invention not explicitly mentioned here 5 may occur to those of skill in the art and such advantages do not depart from the true scope of the invention.

The following Examples merely serve to further illustrate the invention and these examples or any statements made therein should not be construed as a limitation of the scope of the invention.

10

#### EXAMPLE 1

##### Isolation of a cDNA corresponding with Cluster-A

Cluster-A mRNA was reported to be strongly induced after TMV-infection of *Nicotiana tabacum* cv. Samsun NN (Hooft van Huijsduijnen et al, 1986, 15 supra). Screening of a lambda ZAP cDNA library (see EXPERIMENTAL part) of TMV-infected Samsunn NN tobacco plants with a probe derived from PROB40 (a partial Cluster-A cDNA clone) resulted in the isolation of 11 positively hybridizing clones. Restriction enzyme analysis and (partial) sequence analysis revealed that all clones were identical. It was 20 concluded that the clones correspond to Cluster-A cDNA. The nucleotide sequence of one of these clones (cA-3) was determined (EXPERIMENTAL PART) and is depicted in SEQIDNO: 1 and 2. This clone lacks in 7 codons of the 5' part of the open reading frame.

25

#### EXAMPLE 2

##### Cloning of a complete cDNA clone in a bacterial expression vector

The cDNA clone was completed by performing a PCR reaction (EXPERIMENTAL PART) on clone cA-3 with the following primer T7: 30 5'-AATACGACTCACTATAG- (SEQIDNO: 3) and primer p1: 5'-GTTTCCTTCTCCATGGAACTAGTTGCAC (SEQIDNO: 4).

After digestion of the PCR-amplified product with restriction enzymes NcoI and XbaI, electrophoresis and isolation of the fragment from an agarose gel, it was ligated into an intermediate NcoI/XbaI-digested vector pGV84, followed by transformation of *E. coli* DH5 $\alpha$ . The NcoI/XbaI 35 fragment from positive clones resulting from this transformation was subsequently ligated into a NcoI/SalI-digested bacterial expression vector (pJLA602, Medac, Hamburg, Germany) and used to transform *E. coli*

DH5 $\alpha$ . Positive transformants, were screened by hybridization to the labeled clone cA-3 and the presence of the correct size and orientation of the insert was determined by restriction enzyme analysis. The resulting plasmid, pJLA-A53 contains the putative Cluster-A open reading frame (ORF), including codons 20-377, downstream of a tandem arrangement of the repressible p<sub>R</sub> and p<sub>L</sub> promoters of phage lambda and a highly efficient *E. coli* translational initiation region. Finally, plasmid pJLA-A53 was used to transform *E. coli* strain KA1092 (trp65, lon, sFiA-1, malB) (Johnson, B.F. et al., 1977, Mol. Gen. Genet. 157, 91-97) [protease-]. This strain was used to produce Cluster-A protein and to generate antibodies. Strain KA1092, harbouring pJLA-A53, has been deposited at the Centraal Bureau voor Schimmelcultures, on August 12, 1993, accession number CBS 415.93).

15

### EXAMPLE 3

#### Expression of a Clu-A cDNA in *E. coli* and generation of antibodies against the putative Clu-A protein

The Cluster-A cDNA gene, codons 20 - 377 was placed under the control of the lambda p<sub>R</sub> and p<sub>L</sub> promoters (Schauder B. et al., 1987, Gene 56, 279-283), in order to overexpress the gene in *E. coli*. In Figure 5 is shown the overproduction of the putative 40 kDa Cluster-A protein in *E. coli* strain KA1092. After disrupting the cells by sonification, Cluster-A protein aggregates were isolated by centrifugation with the cells debris (Figure 5, lane D). Expression of the putative Cluster-A cDNA gene was induced in KA1092 by switching cultures grown for 3 h at 30°C to a waterbath at 42°C and incubating for another 3 h. Bacteria were collected by centrifugation and dissolved in 1/25 vol. of sample buffer (25 mM Tris, 192 mM glycine, 6 M urea, 2.5 % SDS, 10 % glycerol, 5 %  $\beta$ -mercapto-ethanol). The putative Cluster-A protein produced in *E. coli* was purified and used for the generation of antibodies (EXPERIMENTAL PART).

Figure 5 shows a Western blot analysis of similar protein fractions shown in Figure 5 and demonstrates that the isolated antibodies are specifically directed against the putative Cluster-A protein. A strong signal for Cluster-A protein is found in the total fraction (lane T) and cell debris fraction (lane D) of induced *E. coli* cells which contained

the Cluster-A expression construct. The weak signal found in lane S, indicates that a minor amount of the Cluster-A protein is present in the soluble protein fraction.

After heating for 3 min at 100°C, the samples were electrophoresed 5 in SDS-polyacrylamide gel (EXPERIMENTAL PART) and proteins were visualized by staining with Coomassie Brilliant Blue. Similar gels were stained with icecold 1 M KCl and the regions of the gel containing the overproduced 40 kDa proteins were cut out. After washing in water, the acrylamide was ground and mixed with Freunds complete immunization 10 medium, upon which a suspension with about 200 ng putative Cluster-A was injected subcutaneously into rabbits. After three additional immunizations at two-week intervals with similar suspensions in Freunds incomplete medium, 50 ml blood was collected from the rabbits and the serum fraction was tested for antibodies directed against the putative 15 Cluster-A protein.

#### EXAMPLE 4

##### Isolation and characterization of putative Cluster-A protein from tobacco

The purification of the putative Cluster-A protein from TMV infected 20 tobacco leaves (EXPERIMENTAL PART) was monitored by immunodetection using the antiserum obtained as described in EXAMPLE 3.

The putative Cluster-A protein appeared to absorb to a cation-exchanger S-Sepharose indicating a basic nature of the protein. No cross-reacting proteins were observed in the flow-through of the S-Sepharose column. The 25 putative Cluster-A protein was eluted from the S-Sepharose column in the presence of 75 to 140 mM NaCl. Close examination of the SDS-polyacrylamide gels and immunoblots revealed that two proteins of about the same molecular weight (41 kDa and 43 kDa) cross-reacted to the antiserum. Apparently, both proteins were related. The presence of 30 potential glycosylation sites in both the cDNA sequence (3 potential glycosylation sites) and the gDNA sequence (4 potential glycosylation sites) prompted us to study the binding of the putative Cluster-A protein to Con-A Sepharose. Proteins were, therefore, dialysed to 50 mM Tris, HCl, pH 6.8, containing 1.15 M NaCl and allowed to absorb to Con-A 35 Sepharose. Most of the 41 kDa Cluster-A protein passed through the Con-A column while among the bound proteins the 43 kDa putative Cluster-A protein was present, indicating that this latter protein is at least

partly glycosylated. Con-A Sepharose was not applied in the purification of the putative Cluster-A protein. Instead, chelating Sepharose chromatography was used. The fractions containing the putative Cluster-A protein eluting from the S-Sepharose column were dialysed against 50 mM Tris, HCl buffer pH 8.0, containing 150 mM NaCl and allowed to absorb to a chelating Sepharose column activated with Zn<sup>2+</sup> ions and equilibrated in the above buffer. About 20% of the total amount of protein was retarded by the activated matrix and appeared to consist mainly of Cluster-A protein. Second passage through the chelating Sepharose column resulted in an almost pure protein preparation (Figure 5, lane D). The putative Cluster-A protein was purified to homogeneity by subsequent gelfiltration chromatography. The apparent native molecular weight was between about 40 and 43 kDa.

15

EXAMPLE 5

Elucidation of the amino acid sequence of the putative Cluster-A proteins  
A mixture of both 41 kDa and 43 kDa Cluster-A protein was separated on a 12.5% SDS-polyacrylamide gel and electroblotted to a PVDF membrane. The mixture was then used to determine the N-terminal amino acid sequence by Edman degradation (EXPERIMENTAL PART). However, the N-terminus was blocked and no sequence information was obtained. The presence of a unique site susceptible for acid hydrolysis enabled us to determine an internal sequence from the same material:

25

P-V-N-H-X1-S-G-S-D-X2-I-N-A-X3-I-Q (SEQID NO: 4).

X1= V/I, X2=G, X3=W,

The amino acid preceding this sequence is most probably an Asp (D) residue since the digestion used is specific for Asp-Pro bonds (Landon 1977, sub). Additional sequence information was obtained after cleaving the putative Cluster-A protein with hydroxylamine. This resulted in the formation of two peptides of about the same molecular weight which were blotted together onto a PVDF membrane. Sequencing of this sample yielded the following sequences:

35

G-L-N-Y-P-V-E-S-V-A-R-N-L-N-W (SEQID NO: 5),

and:

S-H-A-Q-L-F-D-P-V-N-H-X-S-G-S-D-G-I-N-A-W-I-Q-A-G-V (SEQID NO: 6), wherein X=I/V.

These peptide sequences showed complete identity to the amino acid sequence deduced from the cDNA clone (Figure 1; SEQIDNO: 1 and 2). It was concluded from these matching data that the two proteins purified from tobacco correspond to the protein produced by *E. coli* KA 1092 transformed with the partial Cluster-A ORF. The peptide sequence, corresponding to codon position 233, showed that two amino acids (V/I) were present at this position in a 1:1 ratio. Possibly two highly identical Cluster-A proteins were purified and differ for one amino acid position in the region sequenced.

Comparison of the Cluster-A protein with matching proteins in a database revealed homology with the bacterial chitinases ChiA and ChiB from *Bacillus circulans* and *Serratia marcescens*, respectively. Although the overall identity of Cluster-A to ChiA (31%) and ChiB (26%) is low, specific regions are highly conserved within all three amino acid sequences, as shown in Figure 4. In addition, homology was found with chitinases from *Streptomyces plicatus*, *Brugia malayi* (nematode), and a secretory protein from *Mus musculus* (Murinae). No structural homology was found with the four distinct classes of plant chitinases (Lawton K. et al, 1992, Plant Mol Biol. 19, 735-743; Collinge, 1993, supra).

#### EXAMPLE 6

##### Enzymatic activities and cellular localization of Cluster-A

The homology of the tobacco Cluster-A protein with bacterial chitinases suggested that this protein might be able to catalyze the hydrolysis of chitin, a  $\alpha$ -1,4-linked polymer of N-acetyl-D-glucosamine. The possibility to purify Cluster-A protein from tobacco material, using the antibody enabled us to test this hypothesis. Cluster-A was tested for chitinase activity. In an endo-chitinase assay, using tritiated chitin as a substrate (Molano J. et al, 1977, Anal. Biochem. 83, 648-656), the Cluster-A protein showed a specific activity of  $40 \pm 6$  cpm per  $\mu$ g protein, which is about 250 to 500 fold lower than the specific activities of the class-I chitinases (Legrand M. et al, 1987, Proc. Natl. Acad. Sci. USA 84, 6750-6754; Sela-Buurlage M.B. et al, 1993, Plant Physiol. 101, 857-863). However, chitinase activity of Cluster-A measured by a colorimetric assay,

using a soluble chitin dye substrate (Wirth and Wolf, 1990, J. Microbiol. Methd. 12, 197-205), was two-fold higher than that of class-I chitinase (Table 1). Testing of Cluster-A for additional enzyme activities indicated that this protein possessed no exo-chitinase activity, chitobiase, 5  $\beta$ -1,3-glucanase or lysozyme activity.

The cellular localization of Cluster-A was analyzed using different leaf fractions of TMV infected tobacco plants, including (a) total leaf fraction, (b) leaf material from which the extracellular fluid (EF) was removed, and (c) the EF-fraction. Western analysis indicated that Cluster-A proteins are 10 predominantly located intracellularly, as shown in Figure 6. A strong signal for Cluster-A protein was found in fraction-a and fraction-b. In the extracellular fluid of TMV infected leaves no Cluster-A protein was found (fraction-c).

15 Table 1

Chitinase activities of Cluster-A proteins  
Chitinase activities (expressed per  $\mu$ g)

Protein	$^3\text{H}$ -chitin assay	CM-chitin assay	
<hr/>			
PR-3a (PR P)	1180 $\pm$ 57 cpm	36.2 $\pm$ 1.10 ODe	
PR-3b (PR Q)	1087 $\pm$ 35 cpm	30.9 $\pm$ 1.90 ODe	
32 kDa (Chi-I)	11471 $\pm$ 347 cpm	1.0 $\pm$ 0.03 ODe	
Cluster-A	40 $\pm$ 6 cpm	2.1 $\pm$ 0.03 ODe	
25 exochitinase #	746 $\pm$ 60 cpm	7.0 $\pm$ 0.34 ODe	

CM-chitin, carboxymethyl-chitin bound to Remazol Brilliant Violet  
& Tritiated chitin substrate (Molano et al, 1977).  
\$ p-nitrophenyl liberation assay, substrate p-nitrophenyl chitobiose (Roberts and  
30 Selitrennikoff, 1988).# exochitinase from S. griseus (Sigma C-1650).

The exochitinase activity of all chitinases was tested with the p-nitrophenyl liberation assay with p-nitrophenyl chitobiose as substrate, according to (Roberts W.K. and Selitrennikoff C.P., 1988, J. Gen. Microbiol. 134, 169-176). For PR-3a, PR-3b, 32 kDa Chi-I, and Cluster-A the amount of 5 liberated p-nitrophenyl was less than 0.05 nmole as opposed to 4.50 + 0.1 nmole for the exo-chitinase (not shown in the table).

All assays were performed at 37°C and pH 6.0. Incubation times were usually 30 minutes but in case of low lysozyme activities these incubations were prolonged. The specific activity is expressed as cpm liberated, and blue 10 substrate liberated measured as an increase of OD, respectively.

#### EXAMPLE 7

##### Antifungal activity of Cluster-A protein in vitro

15 In previous reports it has been demonstrated that class-I chitinases (Chi-I) and  $\beta$ -1,3-glucanases (Glu-I) can inhibit fungal growth in vitro (Mauch et al, 1988; Sela-Buurlage et al, 1993). Both hydrolytic enzymes have been implicated to play an important role in the defense mechanisms of plants against invading fungi. The antifungal activity of Cluster-A was 20 investigated by testing the lysis activity of this protein alone or in combination with Chi-I or Glu-I on different fungi. Cluster-A protein alone caused lysis and growth inhibition of *Trichoderma viride* at a concentration of 12  $\mu$ g/ml. On the phytopathogenic fungus *Alternaria radicina* Cluster-A inhibited fungal growth at 30 to 60  $\mu$ g/ml. Even at high concentrations (600 25  $\mu$ g/ml) no effect was found on *Fusarium solani*. However, Cluster-A protein exhibited on *F. solani* a potent antifungal effect in synergy with the class I  $\beta$ -1,3-glucanase (and chitinase) proteins. The synergistic action of these proteins caused both lysis of pregerminated spores and growth inhibition of *F. solani*, see Figure 7. The Cluster-A protein exhibited no effect on the 30 phytopathogenic fungi *Septoria lycopersici* and *Phytophthora infestans* (data not shown).

Table 2:

Antifungal activity of Cluster-A protein:Lysis activity of Cluster-A on Fusarium solani and Alternaria radicina.

5	Cluster A	Chi-I	Fusarium solani				Alternaria radicina			
			Glu-I		Chi-I		Glu-I			
			μg/well	0	0.1	0.5	0.1	0.5	0.1	0.5
10	0	0	0	0	0	0	5	56	0	0
	2	0	0	0	-	-	26	95	0	0
	5	0	0	5	-	-	34	95	0	0
	10	0	0	5	-	-	95	95	0	0
	10 den.	0	-	-	-	-	-	-	-	-

15 Table 3: Antifungal activity of different protein preparations expressed in % lysis and growth inhibition (GI).

20	protein	protein added (μg/well)	Fusarium solani		Alternaria radicina	
			% lysis	GI	% lysis	GI
	Clu-A	5-10	0%	0	ND	4
	Clu-A	100	0%	0	ND	ND
25	Glucanase	0.1	0%	0	ND	ND
	Clu-A + Gluc.	10:0.1	0%	2-3	ND	ND
	Chitinase	0.1	0%	0	ND	ND
	Clu-A + Chit.	10:0.1	5%	1	ND	ND

Data for the denatured control samples are given in brackets but only in case they differ from 0% lysis and/or Gi=0.

EXAMPLE 8Isolation of a genomic clone encoding Cluster-A protein

A genomic library of *N. tabacum* was screened using the Cluster-A cDNA insert of clone cA-3 as a probe. Three recombinant lambda phages, each 5 containing different restriction fragments hybridizing to the Cluster-A cDNA, were purified. The genomic Cluster-A homologous to cA-3 was amplified by a polymerase chain reaction using specific oligonucleotide primers corresponding to the 5' end coding region LS42:

10 5'-TCTCCGGCAACTAGTTGCAC-3' (SEQIDNO: 10).

and 3'end non-coding region of the Cluster-A cDNA LS42:

5'-GTCATAGTTCACATATCTGTTGCC-3' (SEQIDNO: 11).

Only one of the three phages showed a very strong amplification of specific 15 sequences with these primers and was selected for further analysis. The complete nucleotide sequence of the Cluster-A cDNA, including the deduced primary structure of the Cluster-A protein and sequences of the 5'-flanking and 3'-flanking regions of the gene, are shown in SEQIDNO: 7 and 8.

Comparison of the cDNA clone with the Cluster-A gene revealed that these 20 sequences share a high degree of identity (94%). Within the 5' end upstream region a 'CAAT' motif (position 391) and 'TATAAT' motif (position 428) is found. In the 3' end non-coding region a potential polyadenylation signal (5'-AATAAA-3') is present at position 2971. The coding region of the Cluster-A gene is contained in two exons and yields a precursor protein of 25 377 amino acids. The Cluster-A precursor protein contains a putative signal peptide of 25 amino acids that is involved in transport across the membrane of the endoplasmatic reticulum, as well as 4 potential N-linked glycosylation sites (N-X-S/T). The predicted mature protein has a calculated molecular weight of 39,033 Da. The intervening sequences of 952 bp and 349 30 bp in length are present after codon 151 and 364 respectively. To assess the number of genes encoding Cluster-A like proteins in tobacco, a genomic Southern blot was probed with the cDNA clone. The pattern of bands hybridizing to the probe under stringent conditions shows that Cluster-A is part of a small gene family with at least four different members (Fig. 2). 35 To determine the induction of Cluster-A gene expression, tobacco plants were treated with different stress conditions and analysed on a Northern blot. Samples of different treatments were taken 3 days after inoculation with

TMV, 2 days after wounding, 1 day after ethephon treatment, and 1 day after UV-light irradiation. At these time points maximal expression of the PR genes (PR-1 to PR-5) was reported previously by Brederode and coworkers (1991, Plant Mol. Biol. 17, 1117-1125) and was used here to study Cluster-A gene expression. Figure 3 shows that in non-stressed tobacco leaves there is no detectable expression of the Cluster-A gene. TMV infection of tobacco leaves with ethephon, to produce ethylene, or UV-light resulted both in a high increase of Cluster-A mRNA (Fig. 3, lane E and U). In contrast, a low induction of Cluster-A expression was found after wounding the leaves. These results together indicated that Cluster-A can be classified as a novel pathogenesis-related gene of tobacco.

#### EXAMPLE 9

##### Construction of expression construct and binary vectors

15 The Cluster-A gene is cloned as an approximately 5.5 kb SstI-fragment. The entire open reading frame is located on this fragment occupying about two third of the fragment; the 5' end of the open reading frame starts at about position 453 from one SstI site. Using PCR a BamHI site is introduced just upstream of the 5' end of the ORF as follows. The original sequence (Fig. 1) 20 (position 406) 5'-TTTAAACTCGAAGTCACAAATTAAA-3' (SEQIDNO: 12) (position 432) is modified into (position 406) 5'-TTTAAACTCGGATCCACAAATTAAA-3' (SEQIDNO: 13) (position 432).

Similarly, the EcoRI site in pMOG183 (Fig. 9) is modified into a XbaI-site using the following adaptor sequence in a PCR reaction: 5'-AATTGTCTAGAC-3' 25 (SEQIDNO: 14). The modified cloning vehicle is named pMOG185 and contains the CaMV 35S promoter and the nos-terminator and the BamHI-site for inserting the Cluster-A gene in between. To this end the open reading frame of the Cluster-A gene is cloned as an approximately 5.1 kb BamHI-SstI site, employing the newly created BamHI site of the Cluster-A fragment, into the 30 BamHI site of modified expression vector pMOG185. The Cluster-A gene flanked upstream by the 35S CaMV promoter with double enhancer, and downstream by the nos-terminator, is now located on a XbaI-SstI fragment. This fragment is subsequently cloned into binary vector pMOG800 (Fig. 10). Binary vector pMOG800 contains a plant expressible kanamycin resistance gene between a 35 left and right T-DNA border, as well as a multiple cloning site for insertion of restriction fragments. An *E. coli* strain harbouring this vector has been deposited on August 12, 1993, at the Centraal Bureau voor

Schimmelcultures, Baarn, The Netherlands, No. CBS 414.93) with the aid of the suitable synthetic double-stranded, partially complementary, adapter sequences (e.g. 5'-AGCTCACG-3' and 3'-GTGCTTAA-5'). The binary vector so obtained contains both a essentially constitutively expressible Cluster-A gene and a plant expressible NPTII gene as marker, localized between the left and right T-DNA border sequences.

With the aid of plasmid pRK2013, this pMOG800 derived binary vector is then mobilised from *E.coli* DH5 $\alpha$  into *Agrobacterium tumefaciens* strain MOG101 harbouring a helper plasmid with the Vir-functions. The transconjugants are isolated from these matings on selection medium containing 40 mg/l rifampicin, 250 mg/l spectinomycin, and 100 mg/l kanamycin. (for details see EXPERIMENTAL PART). The *Agrobacterium* cells having received the binary vector are used for the transformation of plants.

15

EXAMPLE 10Transformation of plants and analysis of expressiona. tomato

The transformation of tomato (*Lycopersicon esculentum* cv. Moneymaker) with *Agrobacterium* strains MOG101 harbouring the pMOG800 derived binary vector is performed essentially as described by McCormick et al (1986, Plant Cell Rep. 5, 81-84).

b. tobacco

For the transformation of tobacco use is made of the leaf-disc dip method (1985, Horsch et al, Science 227, 1229-1231). Leaf-discs are cocultivated with *Agrobacterium* strain MOG101 harbouring the binary vector and grown on selection medium with 100 mg/ml kanamycin. The transgenic shoots are regenerated into whole plants and analyzed for expression of the newly introduced genes. For this analysis use is made of Western blots, using the antiserum against the Cluster-A protein and/or glucanase.

c. potato cv. Kardal

Potato tubers must be stored at + 4°C. 7 days before transformation the tubers must be transferred to room temperature.

35 Day 1. Inoculate *Agrobacterium* strain from fresh plate into 10 ml LB + 100 mg/l Kanamycin + 20 mg/l Rifampicillin.

Grow overnight by 29°C

Day 2. Dilute *Agrobacterium* suspension 1:10 in Minimal Medium + 100 mg/l Kanamycin; Grow overnight by 29°C. Pour plates with medium 1.

Day 3. Dilute *Agrobacterium* suspension to OD<sub>600</sub> ± 0.15 in Minimal Medium without antibiotics and grow to OD<sub>600</sub> 0.30-0.70. (this takes about 4 hours).

5 Spin culture down and make a 1:10 dilution in MS<sub>30</sub>R3. Peel the potatoes.

- Wash 1 minute with ethanol 70%.

- Wash 20 minutes in 1:5 diluted Teepol + 0.1% Tween 20.

- Wash once with sterile water.

- Add ± 100 ml 1:1 mixed RK:KI (Blue color must stay for 2 minutes).

10 - Wash the blue color away with sterile water.

- Cut tubers in slices of about 2 mm thickness.

- Cut with bore little disks of the vascular tissue.

- Collect the discs in large petridishes (15 cm) with liquid MS<sub>30</sub>R3 (about 400 per dish).

15 - Dry 40 disks on sterile filterpaper and place them on medium 1 as control (2 dishes).

- Remove MS<sub>30</sub>R3 medium with a pipet and replace it for the *Agrobacterium* mox.

- Replace after 20 minutes the *Agrobacterium* suspension for liquid 20 MS<sub>30</sub>R3.

- Dry disks on sterile filterpaper and transfer them to Petridishes with medium 1 (20 disks per plate).

- Close disks with parafilm and place them in the regeneration room.

Day 4. Pour plates with medium 2.

25 Day 5. Transfer disks to plates with medium 2 (20 disks per plate).

Day 8. Pour plates with medium 3.

Day 9. Transfer disks to plates with medium 3 (10 disks per plate). Make from the disks which are not dipped on day 3 the following controls:

1 Not dipped and NO selection (20 disks, 2 plates)

30 2 Not dipped and WITH selection (20 disks, 2 plates)

Make as additional control:

3 Dipped and NO selection (20 disks, 2 plates)

Transfer the disks every three weeks to fresh medium. After 3 weeks the first shoots are visible.

35 When the shoots are big enough, cut them off and place them in tubes with medium 4 (rooting may take 3 weeks).

Sterile media needed:

## Preparation of basal medium:

- For 1 liter MS<sub>30</sub>R3 100 ml 10 \* MS salts

10 ml 100 \* R3 vitamines

5

30 g sucrose

8 g Diachin agar

Adjust pH 5.8 with KOH and sterilize 20' 110°C.

## Preparation of medium for potato stock collection:

- For 1 liter 1/2 MS<sub>20</sub>R3: 50 ml 10 \* MS salts

10

5 ml 100 \* MS vitamines

30 g Sucrose

8 g Daichin agar

Adjust pH 5.8 with KOH and sterilize 20' 110°C.

## Basal medium with proper hormones and antibiotics:

15 1 MS<sub>30</sub>R3 + 3.5 mg/l Zeatin Riboside 500 µl stock solution (3.5 mg/ml) per 500 ml;

0.03 mg/l I.A.A. 15 µl stock solution (1.0 mg/ml) per 500 ml.

2 MS<sub>30</sub>R3 + 3.5 mg/l Zeatin Riboside,

20 0.03 mg/l I.A.A.,

200 mg/l Cefotaxime, 500 µl stock solution (200 mg/ml) per 500 ml,  
100 mg/l Vancomycin

500 µl stock solution (100 mg/ml) per 500 ml

25 3 MS<sub>30</sub>R3 + 3.5 mg/l Zeatin Riboside

0.03 mg/l I.A.A.

200 mg/l Cefotaxime

100 mg/l Vancomycin

100 mg/l Kanamycin 500 µl stock solution (100 mg/ml) per 500 ml

30

## Stock collection medium with proper hormones and antibiotics:

4 1/2 MS<sub>30</sub>R3 + 100 mg/l Cefotaxime

50 mg/l Vancomycin

35

100 mg/l Kanamycin

d. analysis of gene expression

Transgenic plants may be analysed for the Cluster-A protein on Western blots using an anti-Cluster A antiserum. If mRNA levels are tested on Northern blots a suitable cDNA fragment from Cluster-A is used as a probe.

5 Plants with enhanced production of the cluster-A protein are tested for enhanced resistance levels against fungi.

Plants which exhibit simultaneously enhanced levels of Cluster-A and B-1,3-glucanase are expected to possess a higher resistance against fungal infection.

10

EXPERIMENTALPolymerase chain reaction

DNA amplification was performed during 35 cycles of sequential incubations at 94°C for 0.5 min, 50°C for 2 min and 72°C for 1.5 min, in a 50 µl

15 reaction mixture, containing 1 ng of Cluster-A DNA, 0.2 µM of T7 primer, 0.2 µM of primer p1 and 2 units of *Thermus aquaticus* DNA polymerase

(Perkin-Elmer Cetus, Gouda, The Netherlands). Part of the reaction products (4%) was electrophoresed in an agarose gel, to confirm the synthesis of a DNA fragment with the correct size. The remainder of the PCR DNA products

20 was precipitated with ethanol and washed to remove excess of salts.

Synthesis and analysis of cDNA libraries

Isolation of poly(A)+-RNA from TMV-infected tobacco, the synthesis of cDNA and the construction of a cDNA library, unidirectionally cloned in the

25 lambda vector Uni-ZAP XR, was performed according to the manufacturer's instructions (Stratagene, La Jolla Ca.). Recombinant phages were screened using the <sup>32</sup>P-labelled insert of cDNA clone PROB40 as a probe (Hooft van Huijsduijnen R.A.M. et al, 1986, EMBO J. 5, 2057-2061). After rescue of the cDNA-containing pBluescript plasmids from the isolated lambda phage by

30 coinfection with helper phage R408 (Stratagene) the cDNA insert of Cluster-A was subcloned in M13 derivatives and sequenced using the M13 primer and a primer based on the cDNA sequence obtained with the M13 primer.

Alternatively, the cDNA was directly sequenced from denatured plasmid DNA, using T3 or T7 primers (Promega, Madison WI; Chen & Seeburg, 1985, DNA 4, 35 165-170)

Protein purification

Proteins were extracted from tobacco leaves, 7 days after infection with tobacco mosaic virus and desalted by passage through a G-25 column, equilibrated in 40 mM NaOAc, pH 5.2 (Woloshuk C.P. et al, 1991, *The Plant Cell* 3, 619-628). The desalted protein solution was left overnight on ice before centrifuging 50 min at 20,000 g. The resulting supernatant was loaded onto a S-Sepharose (fast flow, Pharmacia) column (5 x 5 cm) equilibrated in 40 mM NaOAc, pH 5.2. Adsorbed proteins were eluted with a linear salt gradient from 0 to 0.3 M NaCl in the above buffer. Every third fraction was analyzed by immunoblotting and the Cluster-A containing fractions were pooled and dialysed to 50 mM Tris, HCl pH 8.0. This solution was passed through a chelating Sepharose column (HR 10/2; Pharmacia) activated by a slightly acidic solution (50 ml) of 0.1 M ZnCl<sub>2</sub> (pH = 5.4). The column was extensively washed with water (500 ml) and then equilibrated in the above Tris-buffer prior to use. The Cluster-A containing pool was loaded in several runs each containing 3 to 4 mg protein. The retarded protein peak was pooled and subjected to chelating Sepharose chromatography again. Although a tighter binding was observed after activation of the chelating sepharose column with Cu<sup>2+</sup>-ions, purification under these conditions was less effective (data not shown). The final purification step consisted of gelfiltration through a Superdex-75 column (HR 10/30; Pharmacia equilibrated in 50 mM KHPO<sub>4</sub> buffer, pH 7.0, containing 200 mM NaCl. Gelfiltration was carried out at a flow rate of 0.5 ml per minute and fractions of 0.5 ml were collected. The fractions were analyzed by SDS-PAA gelectrophoresis and immunoblotting.

SDS-gelectrophoresis, immunoblotting and sequence determinations

Protein samples were separated on 12.5 % SDS-PAA gels as described by Laemmli (Laemmli U.K., 1970, *Nature* 227, 680-685). Immunoblotting and detection was performed according to the ECL Western blotting protocol provided by AMersham, UK. The antiserum to the PR-4a,b homologue from tomato (PR-P2) was kindly provided by Matthieu Joosten, Wageningen, The Netherlands. For immunodetection the antiserum to putative cluster-A protein was used in a 1:2000 dilution.

For sequencing purposes the protein was separated as described by Moos (Moos M. et al, 1988, *J. Biol. Chem.* 263, 6005-6008) and electroblotted onto PVDF membrane as described by Matsudaira et al. (Matsudaira et al, 1987, *J.*

Biol. Chem. 262, 10035-10038). Proteins were visualized by Coomassie Brilliant Blue R-250 staining (Matsudaira et al, 1987, *supra*). Since the putative Cluster-A protein was N-terminally blocked it was acid hydrolysed on the PVDF membrane as described by Landon (Landon M., 1977, Methods of Enz. 47, 145-149). The digest (consisting of two peptides) was sequenced by Eurosequence, Groningen, The Netherlands, using Edman degradation on an Applied Biosystems 477A protein sequencer.

#### Enzyme assays

10 Chitinase activity measurements were routinely carried out as described by Molano et al (1977, *supra*). Alternative assays like the blue substrate assay described by Wirth and Wolf (Wirth S.J. and Wolf G.A., 1990, J. Microbiol. Meth. 12, 197-205), the p-nitrophenyl liberation assay described by Roberts and Selitrennikoff (Roberts W.K. and Selitrennikoff C.P., 1988, J. Gen. Microbiol. 134, 169-176) and the 4-methylumbelliflferone liberation assay described by McCreath and Gooday (McCreath K.J. and Gooday G.W., 1991, J. Microbiol. Meth. 14, 229-237) were also used.  
Lysozyme activity was determined in 50 mM KPO<sub>4</sub> buffer, pH 6.0 as described by Selsted and Martinez (1980, Anal.Biochem. 109, 67-70).

20

#### Chitinase assays

Bacteria induced to express the cA-3 protein and control bacteria were collected from 10 ml cultures by centrifugation and the pellets were resuspended in 1 ml TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). After 25 sonification of the suspensions were stored at -20°C. Before assaying for chitinase activity, 1 % Triton X-100 was added. Total protein extracts of healthy or TMV-infected tobacco leaf was obtained by homogenization in 5 ml of TBS. The homogenates were briefly centrifugated to remove the insoluble material and stored at -20°C. Extracellular proteins were obtained by vacuum infiltration of leaves with H<sub>2</sub>O (nanopure). The infiltrated leaves were placed in a syringe and centrifuged for 5 min at 3000 rpm in a table top centrifuge. The colourless washing containing the extracellular proteins was stored at -20°C. Chitin-hydrolysing activity was assayed in 200 µl reaction mixtures containing 50 µl of thoroughly washed <sup>3</sup>H-labeled chitin 30 suspension, 50 µl bacterial or plant extract and 100 µl 50 mM potassium phosphate, pH 6.4. after incubation for 30 min at 30°, 0,5 ml of 10 % trichloroacetic acid was added, the mixture was centrifugated to remove 35 suspension, 50 µl bacterial or plant extract and 100 µl 50 mM potassium phosphate, pH 6.4. after incubation for 30 min at 30°, 0,5 ml of 10 % trichloroacetic acid was added, the mixture was centrifugated to remove

precipitated material and 0,5 ml of the supernatant was removed and filtered through glass-wool. Solubilized radioactivity was measured in a liquid scintillation counter.

5 RNA blot and DNA blot analysis

Total RNA from non-stressed or stressed tobacco was extracted from frozen leaf-tissue by homogenization in extraction buffer (1 M Tris-HCl, 0.1 M LiCl, 10 mM EDTA, 1% SDS, pH 9.0). The homogenate was extracted with phenol and chloroform and the RNA was precipitated with 2 M LiCl. The RNA was 10 electrophoresed in 1.5 % agarose gels after glyoxylation and blotted to nylon membranes (Genescreen, New England Nuclear or Hybond-N, Amersham). Tobacco genomic DNA was isolated and digested, electrophoresed and blotted as described (Cornelissen et al, 1987, Nucl. Acids Res. 15, 6799-6811). Hybridization of tobacco nucleic acids was performed with the <sup>32</sup>P-labelled 15 cDNA insert of Cluster-A in 5x SSC, 2% SDS, 50% formamide, 100 µg/ml herring sperm DNA at 42°C (1x SDS at 50°C and autoradiographed.

Obtention of Agrobacterium strain MOG101

A helper plasmid conferring the *Agrobacterium tumefaciens* virulence 20 functions derived from the octopine Ti-plasmid pTiB6 was constructed, MOG101. MOG101 is an *Agrobacterium tumefaciens* strain carrying a non-oncogenic Ti-plasmid from which the entire T-region was substituted by a bacterial Spectinomycin resistance marker from transposon Tn 1831 (Hooykaas et al, 1980 Plasmid 4, 64-75).

25 The Ti-plasmid pTiB6 contains two adjacent T-regions, TL (T-left) and TR (T-right). To obtain a derivative lacking the TL- and TR-regions, we constructed intermediate vector pMOG579. Plasmid pMOG621 is a pBR322 derivative, which contains the 2 Ti-plasmid fragments that are located to the left and right, outside the T-regions (Figure 2). In pMOG579 the 2 30 fragments (shown in dark) were separated by a 2.5 kb BamHI - HindIII fragment from transposon Tn1831 (Hooykaas et al, 1980 Plasmid 4, 64-75) carrying the spectinomycin resistance marker (Figure 2). The plasmid was introduced into *Agrobacterium tumefaciens* strain LBA1010 [C58-C9 (pTiB6) = a cured C58 strain in which pTiB6 was introduced (Koekman et al (1982), 35 Plasmid 2, 119-132) by triparental mating from *E. coli*, using HB1018 (pRK2013) as a helper. Transconjugants were selected for resistance to Rifampicin (20 mg/l) and spectinomycin (250 mg/l). A double recombination

between pMOG579 and pTiB6 resulted in loss of carbenicillin resistance (the pBR322 marker) and deletion of the entire T-region. Of 5000 spectinomycin resistant transconjugants replica plated onto carbenicillin (100 mg/l) 2 were found sensitive. Southern analysis showed that a double crossing over 5 event had deleted the entire T-region (not shown). The resulting strain was called MOG101. This strain and its construction is analogous to strain GV2260 (Deblaere et al 1985, Nucl. Acid Res. 13, 4777-4788). (MOG101 is described in more detail in Hood E., Gelvin S.B., Melchers L.S. and Hoekema A., 1993, Transgene Research 2, 208-218 and is freely available from MOGEN 10 International N.V. upon request.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: MOGEN International N.V.
  - (B) STREET: Einsteinweg 97
  - (C) CITY: LEIDEN
  - 10 (D) STATE: Zuid-Holland
  - (E) COUNTRY: The Netherlands
  - (F) POSTAL CODE (ZIP): NL-2333 CB
  - (G) TELEPHONE: 31.71.258282
  - (H) TELEFAX: 31.71.221471
- 15
- (A) NAME: Rijksuniversiteit te Leiden
  - (B) STREET: Stationsweg 46
  - (C) CITY: LEIDEN
  - (D) STATE: Zuid-Holland
  - 20 (E) COUNTRY: Netherlands
  - (F) POSTAL CODE (ZIP): NL-2312 AV

(ii) TITLE OF INVENTION: Plant chitinases, DNA coding therefor and plants containing same

25

(iii) NUMBER OF SEQUENCES: 14

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- 30 (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## 35 (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1253 base pairs
- (B) TYPE: nucleic acid
- 40 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

45 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Nicotiana tabacum
- (B) STRAIN: Samsun NN
- 50 (D) DEVELOPMENTAL STAGE: TMV-induced

(vii) IMMEDIATE SOURCE:

- (B) CLONE: Cluster-A

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 14..1126
- (D) OTHER INFORMATION: /partial

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

	CGAGTTTTT TTT TTT TCT ATT ATT TTC TCA TGT TTC CTT CTC CGG CAA	49
10	Phe Ser Ile Ile Phe Ser Cys Phe Leu Leu Arg Gln	
	1 5 10	
	CTA GTT TGC ACA AAT AGC CAA AAT GTT ATT AAG GGA GGG TAC TGG TTT	97
	Leu Val Cys Thr Asn Ser Gln Asn Val Ile Lys Gly Gly Tyr Trp Phe	
15	15 20 25	
	AAG AAC AGT GGA TTA GCA TTA AAC AAC ATA GAC TCA ACA CTT TTC ACT	145
	Lys Asn Ser Gly Leu Ala Leu Asn Asn Ile Asp Ser Thr Leu Phe Thr	
	30 35 40	
20	CAT CTA TTT TGT GCA TTT GCT GAT CTT AAT CCA CAA TCA AAT CAG TTA	193
	His Leu Phe Cys Ala Phe Ala Asp Leu Asn Pro Gln Ser Asn Gln Leu	
	45 50 55 60	
25	ATC ATT TCG CCA GAA AAT CAA GAT TCA TTC AGC CAA TTT ACA AGT ACA	241
	Ile Ile Ser Pro Glu Asn Gln Asp Ser Phe Ser Gln Phe Thr Ser Thr	
	65 70 75	
30	GTT CAA AGG AAA AAT CCT TCA GTC AAG ACT TTC TTG TCT ATA GCT GGA	289
	Val Gln Arg Lys Asn Pro Ser Val Lys Thr Phe Leu Ser Ile Ala Gly	
	80 85 90	
35	GGA AGA GCT GAT ACA ACT GCC TAT GGA ATT ATG GCT AGA CAA CCA AAT	337
	Gly Arg Ala Asp Thr Thr Ala Tyr Gly Ile Met Ala Arg Gln Pro Asn	
	95 100 105	
	TCA AGA AAA AGT TTT ATT GAT TCA TCA ATA AGA TTG GCT AGA CAA TTT	385
	Ser Arg Lys Ser Phe Ile Asp Ser Ser Ile Arg Leu Ala Arg Gln Phe	
	110 115 120	
40	GGA TTT CAT GGC CTT GAT CTT GAT TGG GAA TAT CCA TTA TCA GCT ACA	433
	Gly Phe His Gly Leu Asp Leu Asp Trp Glu Tyr Pro Leu Ser Ala Thr	
	125 130 135 140	
45	GAT ATG ACA AAC TTA GGG ATC CTT TTG AAT GAG TGG CGC ACC GCT ATC	481
	Asp Met Thr Asn Leu Gly Ile Leu Leu Asn Glu Trp Arg Thr Ala Ile	
	145 150 155	
50	AAC ATG GAG GCG AGA AAT TCC GGC AGG GCG GCA CTG CTT CTC ACG GCG	529
	Asn Met Glu Ala Arg Asn Ser Gly Arg Ala Ala Leu Leu Leu Thr Ala	
	160 165 170	

	GCG GTT TCC TAC TCA CCC CGA GTC AAT GGA TTG AAC TAC CCA GTT GAA Ala Val Ser Tyr Ser Pro Arg Val Asn Gly Leu Asn Tyr Pro Val Glu 175 180 185	577
5	TCG GTG GCA AGA AAC TTA AAC TGG ATT AAC CTT ATG GCA TAT GAC TTC Ser Val Ala Arg Asn Leu Asn Trp Ile Asn Leu Met Ala Tyr Asp Phe 190 195 200	625
10	TAT GGA CCA AAT TGG TCA CCA TCA CAA ACC AAT TCA CAT GCA CAA TTA Tyr Gly Pro Asn Trp Ser Pro Ser Gln Thr Asn Ser His Ala Gln Leu 205 210 215 220	673
15	TTT GAT CCT GTG AAC CAT ATT AGT GGA AGC GAT GGA ATT AAT GCA TGG Phe Asp Pro Val Asn His Ile Ser Gly Ser Asp Gly Ile Asn Ala Trp 225 230 235	721
20	ATT CAA GCT GGT GTT CCA ACA AAA AAA TTG GTA CTT GGA ATT CCA TTT Ile Gln Ala Gly Val Pro Thr Lys Lys Leu Val Leu Gly Ile Pro Phe 240 245 250	769
25	TAT GGC TAT GCG TGG CGA TTG GTT AAC CCG AAT ATC CAC GAT CTT AGA Tyr Gly Tyr Ala Trp Arg Leu Val Asn Pro Asn Ile His Asp Leu Arg 255 260 265	817
30	GCA CCT GCC GGC GGA AAA TCA AAT GTA GGT GCG GTC GAT GAT GGG TCG Ala Pro Ala Ala Gly Lys Ser Asn Val Gly Ala Val Asp Asp Gly Ser 270 275 280	865
35	ATG ACT TAT AAC AGA ATT AGA GAT TAT ATA GTG CAG AGT CGC GCC ACA Met Thr Tyr Asn Arg Ile Arg Asp Tyr Ile Val Gln Ser Arg Ala Thr 285 290 295 300	913
40	ACT GTG TAT AAT GCT ACT ATT GTT GGA GAT TAT TGT TAC TCT GGA AGT Thr Val Tyr Asn Ala Thr Ile Val Gly Asp Tyr Cys Tyr Ser Gly Ser 305 310 315	961
45	AAT TGG ATT AGC TAT GAT GAT ACT CAA AGT GTT AGA AAT AAG GTT AAT Asn Trp Ile Ser Tyr Asp Asp Thr Gln Ser Val Arg Asn Lys Val Asn 320 325 330	1009
50	TAT GTT AAA GGT AGA GGA TTG CTA GGT TAC TTT GCA TGG CAC GTT GCA Tyr Val Lys Gly Arg Gly Leu Leu Gly Tyr Phe Ala Trp His Val Ala 335 340 345	1057
	GGG GAT CAA AAT TGG GGA CTT TCT CGT ACA GCT TCA CAA ACA TGG GGA Gly Asp Gln Asn Trp Gly Leu Ser Arg Thr Ala Ser Gln Thr Trp Gly 350 355 360	1105
	GTG TCA TCT CAA GAG ATG AAG TGATGGATTA CGTAATTGTG TGTGTCAAGT Val Ser Ser Gln Glu Met Lys 365 370	1156

ATACTACTTA TAATAAGGCA ACAGATATGT GAACTATGAC ATAAATAAAT AAACAATAAA 1216  
 TTGTGGTCTC CAAAAAAA AAAAAAAA AAAAAAA 1253

5

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 371 amino acids
- 10 (B) TYPE: amino acid
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

15

Phe Ser Ile Ile Phe Ser Cys Phe Leu Leu Arg Gln Leu Val Cys Thr  
 1 5 10 15

20

Asn Ser Gln Asn Val Ile Lys Gly Gly Tyr Trp Phe Lys Asn Ser Gly  
 20 25 30

Leu Ala Leu Asn Asn Ile Asp Ser Thr Leu Phe Thr His Leu Phe Cys  
 35 40 45

25

Ala Phe Ala Asp Leu Asn Pro Gln Ser Asn Gln Leu Ile Ile Ser Pro  
 50 55 60

Glu Asn Gln Asp Ser Phe Ser Gln Phe Thr Ser Thr Val Gln Arg Lys  
 65 70 75 80

30

Asn Pro Ser Val Lys Thr Phe Leu Ser Ile Ala Gly Gly Arg Ala Asp  
 85 90 95

35

Thr Thr Ala Tyr Gly Ile Met Ala Arg Gln Pro Asn Ser Arg Lys Ser  
 100 105 110

Phe Ile Asp Ser Ser Ile Arg Leu Ala Arg Gln Phe Gly Phe His Gly  
 115 120 125

40

Leu Asp Leu Asp Trp Glu Tyr Pro Leu Ser Ala Thr Asp Met Thr Asn  
 130 135 140

Leu Gly Ile Leu Leu Asn Glu Trp Arg Thr Ala Ile Asn Met Glu Ala  
 145 150 155 160

45

Arg Asn Ser Gly Arg Ala Ala Leu Leu Leu Thr Ala Ala Val Ser Tyr  
 165 170 175

50

Ser Pro Arg Val Asn Gly Leu Asn Tyr Pro Val Glu Ser Val Ala Arg  
 180 185 190

Asn Leu Asn Trp Ile Asn Leu Met Ala Tyr Asp Phe Tyr Gly Pro Asn  
 195 200 205

Trp Ser Pro Ser Gln Thr Asn Ser His Ala Gln Leu Phe Asp Pro Val  
 210 215 220

Asn His Ile Ser Gly Ser Asp Gly Ile Asn Ala Trp Ile Gln Ala Gly  
 5 225 230 235 240

Val Pro Thr Lys Lys Leu Val Leu Gly Ile Pro Phe Tyr Gly Tyr Ala  
 245 250 255

10 Trp Arg Leu Val Asn Pro Asn Ile His Asp Leu Arg Ala Pro Ala Ala  
 260 265 270

Gly Lys Ser Asn Val Gly Ala Val Asp Asp Gly Ser Met Thr Tyr Asn  
 275 280 285

15 Arg Ile Arg Asp Tyr Ile Val Gln Ser Arg Ala Thr Thr Val Tyr Asn  
 290 295 300

Ala Thr Ile Val Gly Asp Tyr Cys Tyr Ser Gly Ser Asn Trp Ile Ser  
 305 310 315 320

20 Tyr Asp Asp Thr Gln Ser Val Arg Asn Lys Val Asn Tyr Val Lys Gly  
 325 330 335

Arg Gly Leu Leu Gly Tyr Phe Ala Trp His Val Ala Gly Asp Gln Asn  
 25 340 345 350

Trp Gly Leu Ser Arg Thr Ala Ser Gln Thr Trp Gly Val Ser Ser Gln  
 355 360 365

30 Glu Met Lys  
 370

(2) INFORMATION FOR SEQ ID NO: 3:

35 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 29 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA  
 (iii) HYPOTHETICAL: YES

45 (ix) FEATURE:  
 (A) NAME/KEY: misc\_feature  
 (B) LOCATION: 1..29  
 (D) OTHER INFORMATION: /function= "pcr-primer"

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTTCCTTCT CCATGGAAC AGTTGCAC

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- 15 (A) ORGANISM: Nicotiana tabacum  
(B) STRAIN: Samsun NN  
(D) DEVELOPMENTAL STAGE: TMV-induced  
(F) TISSUE TYPE: Leaf

20 (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 5  
(D) OTHER INFORMATION: /label= X  
/note= "residue is Val or Ile"

25 (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 10  
(D) OTHER INFORMATION: /label= x  
/note= "probably Gly"

(ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 14  
35 (D) OTHER INFORMATION: /label= x  
/note= "unknown residue"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Pro Val Asn His Xaa Ser Gly Ser Asp Xaa Ile Asn Ala Xaa Ile Gln  
1 5 10 15

## 45 (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 15 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- 5 (A) ORGANISM: Nicotiana tabacum  
(D) DEVELOPMENTAL STAGE: TMV-induced  
(F) TISSUE TYPE: leaf

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Leu Asn Tyr Pro Val Glu Ser Val Ala Arg Asn Leu Asn Trp  
1 5 10 15

15 (2) INFORMATION FOR SEQ ID NO: 6:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

25 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Nicotiana tabacum  
(D) DEVELOPMENTAL STAGE: TMV-induced  
(F) TISSUE TYPE: leaf

30 (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 12  
(D) OTHER INFORMATION: /label= x  
35 /note= "both Val and Ile occur"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ser His Ala Gln Leu Phe Asp Pro Val Asn His Xaa Ser Gly Ser Asp  
40 1 5 10 15

Gly Ile Asn Ala Trp Ile Gln Ala Gly Val  
20 25

45 (2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 3155 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Nicotiana tabacum
- 5 (B) STRAIN: Samsun NN

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 454..907

10

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 1859..2497

15

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 2847..2884

20

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 908..1858

25

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 2498..2846

30

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(454..907, 1859..2497, 2847..2884)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAGCTCTGTT AATAATTTG ACGTAAC TAG TCATCAGGTC GCAGTTCAAG TCATCAGGTA	60
35 GCAGTTCAAG TCATGAAAAT AGTCTCTTGC AGGTAAGGTT GCGTACAACA GATCCTTGTG	120
GTCCGGCCCT TTCCGAAC TT CGCTTGTAGC TGGAGCTTAG TCACCGGAAT ACCCGTTGTT	180
40 CCGTTAATAA CAAGAACACA TACAAGACGA TTTGCTAACAA AAAATATATA TATAAACCAA	240
AAGTGTAACG ATGATAAAAT TGCACAATTG TGAAGGATAT ACAGGAAAAT TTTGACCTTT	300
TCTCTATTAA GAAATATTGA GAGATTCAAC CACTTCTAA TGGAGTAGTA AATGTATCGC	360
45 AAACCTAAAG CCGCCGGTAG ACAGTAAACT CAAATCTTCA AACTTATTAA AACTCGAAGT	420
CACAAATTAA ATAAGCTATC AACCTTCAAT TTA ATG GCT AAT TCT GTC ACT CTT	474
Met Ala Asn Ser Val Thr Leu	
50 1 5	
TTC GCC ATT ATT TTC TCA TGT TTC CTC CTC CAG CAA CTA GTT TGC ACA	522
Phe Ala Ile Ile Phe Ser Cys Phe Leu Leu Gln Gln Leu Val Cys Thr	
10 15 20	

AAT AGC CAA AAT GTT AAG GGA GGA TAC TGG TTT AAG GAC AGT GGA TTA Asn Ser Gln Asn Val Lys Gly Gly Tyr Trp Phe Lys Asp Ser Gly Leu 25 30 35	570
5 GCA TTA AAC AAC ATA GAT TCA ACA CTT TTC ACT CAT CTA TTT TGT GCA Ala Leu Asn Asn Ile Asp Ser Thr Leu Phe Thr His Leu Phe Cys Ala 40 45 50 55	618
10 TTT GCC GAT CTT AAT CCT CAA TTA AAT CAG TTA ATT ATT TCG CCG GAA Phe Ala Asp Leu Asn Pro Gln Leu Asn Gln Leu Ile Ile Ser Pro Glu 60 65 70	666
15 AAT CAA GAT TCA TTC AGG CAA TTT ACA AGT ACA GTT CAA AGG AAA AAT Asn Gln Asp Ser Phe Arg Gln Phe Thr Ser Thr Val Gln Arg Lys Asn 75 80 85	714
20 CCT TCG GTC AAG ACT TTC TTG TCT ATA GCT GGA GGA AGA GCT AAT TCA Pro Ser Val Lys Thr Phe Leu Ser Ile Ala Gly Gly Arg Ala Asn Ser 90 95 100	762
25 ACT GCC TAT GGA ATT ATG GCT AGA CAA CCA AAT TCA AGA AAA AGT TTT Thr Ala Tyr Gly Ile Met Ala Arg Gln Pro Asn Ser Arg Lys Ser Phe 105 110 115	810
30 ATT GAT TCA TCA ATA AGA TTG GCT AGA CAA TTA GGA TTT CAT GGC CTT Ile Asp Ser Ser Ile Arg Leu Ala Arg Gln Leu Gly Phe His Gly Leu 120 125 130 135	858
35 GTATTATTGA CAAACATATA TTTCTTCAT TTGTTTACA CATATGGATG GCCCTTACAT GAATTATTT TTGGACAAAT ATAGCTAGTT GTGAATTAG GAGAGGCAAT CGGGCGGGTC	967 1027
40 GTGTGGATA TGGGATGGAT TGAAAATGGA TAATGAAAAA ATGGATAAAG TATCCGACCC	1087
45 GATTCAATT TAATACGGAT AATAAACCGG TCCATGACTT CTTGAATATG ATCCCTTTG GGAGAATTTC TATTTCCCA AACTTACCCC CAATTGAAAG CTTTATAAAT GCAAAAGTTA AACTCATTAG TTATCCATTG ATTATCCATT TTCCAAATGG ATAATATGAT TCTTATTAT ATTTGACCCG TTTTAAAAAA GTTCATTATC CAACCCATT TTAGTGGATA ATATGGGTGG	1147 1207 1267 1327
50 TTAATTCTTT TCTTTAACCC ATTTTGCCAT CACTAGTTGT GATATTGAA CTCTCATCTG TCTGTTAGA TATTATATTG AATTATGTGA CAACAGTATT TGAAATTAGA AATTATTATA CACATAATAT ATTTTATTT ACTTAATTAT CATATCTCTG ACTGCAATAC TAGGTTCTTG ATACTAGACA CATTATATGT AACTTTGCA AATTATGGGT CTATGATATT TTCGAAAATA	1387 1447 1507 1567

	ATAACAATTT TTTCACTCTC CTCACAGTTA TATATAATCT CGAAAGGGGA AAGATCCCTC	1627
5	AGAGAATCAC TCGATTATAT TGACAAGTTC AAAAAGTGA TCATAATATG ATGGCGATTG	1687
	CAAGCAGGCC CCCATCATCT CATAACAACT GATAGGTTTC GTTAAACCTG CTCATTATAT	1747
	CCATATGTCT ACTATGATAC CATACAATAT GAATTGTGTA ATCACTCCAT CTAGCTAAC	1807
10	ATTTAAACTG TTGAAGAGTA CACTTTATT TACTTACCTG TTAATTAAAC A GG ACC	1863
	Gly Thr	
15	CTT TTG AAT GAG TGG CGC ACC GCT ATC AAC ACG GAG GCG AGA AAT TCC Leu Leu Asn Glu Trp Arg Thr Ala Ile Asn Thr Glu Ala Arg Asn Ser 155 160 165	1911
20	GGT AGG GCG GCA CTA CTT CTC ACG GCG GCG GTT TCC AAC TCA CCC CGA Gly Arg Ala Ala Leu Leu Leu Thr Ala Ala Val Ser Asn Ser Pro Arg 170 175 180 185	1959
25	GTC AAT GGG TTG AAC TAC CCA GTT GAA TCA TTG GCA AGA AAC TTA GAC Val Asn Gly Leu Asn Tyr Pro Val Glu Ser Leu Ala Arg Asn Leu Asp 190 195 200	2007
	TGG ATT AAC CTT ATG GCC TAT GAT TTC TAT GGA CCA AAT TGG TCA CCA Trp Ile Asn Leu Met Ala Tyr Asp Phe Tyr Gly Pro Asn Trp Ser Pro 205 210 215	2055
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50	GAT TAT ATA GTG GAG AGT CGC GCC ACG ACT GTG TAT AAC GCT ACC ATT Asp Tyr Ile Val Glu Ser Arg Ala Thr Thr Val Tyr Asn Ala Thr Ile 300 305 310	2343

GTT GGA GAT TAT TGT TAC TCT GGT AGT AAT TGG ATT AGC TAT GAT GAT Val Gly Asp Tyr Cys Tyr Ser Gly Ser Asn Trp Ile Ser Tyr Asp Asp 315 320 325	2391
5 ACT CAA ACT GTT AGA AAT AAG GTT AAT TAT GTT AAA GGT AGA GGA TTG Thr Gln Thr Val Arg Asn Lys Val Asn Tyr Val Lys Gly Arg Gly Leu 330 335 340 345	2439
10 CTT GGT TAT TTT GCA TGG CAC GTT GCA GGG GAT CAA AAT TGG GGA CTT Leu Gly Tyr Phe Ala Trp His Val Ala Gly Asp Gln Asn Trp Gly Leu 350 355 360	2487
15 TCT CGT ACA G GTTAGACGTA GTTTTCCTT GTTTTTTTT TTAATTCTGG Ser Arg Thr	2537
TATTTATATT TTTGTATGGA TAACACGTA TTTTGTGAA TACATAATTG TCAAAATACA	2597
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25 ATTATTATTA TTATTATTAT TATTATTATT ATTATTATTA TTTACATTTC CCCCCACTTGT	2837
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30 TGATGGATTA CGTAATTGTG TTTGTCAAGT ATCCTACTTA TAATAAGGTG ACAGATATGT	2944
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TAATATATTT CTTCCCCCTT TGGAGTACAT TAGTTTATT ATTGTTATTA TTGTTGTTGT	3064
35 TGTTGTTGT ACTAATAATA TATTCTTCG TTCCAAAATA GTTGATGCTT TTTGTTTTC	3124
GATATTATTT TCTCGTTTC AATTAATTAA A	3155

## 40 (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 377 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

50 Met Ala Asn Ser Val Thr Leu Phe Ala Ile Ile Phe Ser Cys Phe Leu  
 1 5 10 15

44

Leu Gln Gln Leu Val Cys Thr Asn Ser Gln Asn Val Lys Gly Gly Tyr  
20 25 30

Trp Phe Lys Asp Ser Gly Leu Ala Leu Asn Asn Ile Asp Ser Thr Leu  
5 35 40 45

Phe Thr His Leu Phe Cys Ala Phe Ala Asp Leu Asn Pro Gln Leu Asn  
50 55 60

10 Gln Leu Ile Ile Ser Pro Glu Asn Gln Asp Ser Phe Arg Gln Phe Thr  
65 70 75 80

Ser Thr Val Gln Arg Lys Asn Pro Ser Val Lys Thr Phe Leu Ser Ile  
85 90 95

15 Ala Gly Gly Arg Ala Asn Ser Thr Ala Tyr Gly Ile Met Ala Arg Gln  
100 105 110

Pro Asn Ser Arg Lys Ser Phe Ile Asp Ser Ser Ile Arg Leu Ala Arg  
20 115 120 125

Gln Leu Gly Phe His Gly Leu Asp Leu Asp Trp Glu Tyr Pro Leu Ser  
130 135 140

25 Ala Ala Asp Met Thr Asn Leu Gly Thr Leu Leu Asn Glu Trp Arg Thr  
145 150 155 160

Ala Ile Asn Thr Glu Ala Arg Asn Ser Gly Arg Ala Ala Leu Leu Leu  
165 170 175

30 Thr Ala Ala Val Ser Asn Ser Pro Arg Val Asn Gly Leu Asn Tyr Pro  
180 185 190

Val Glu Ser Leu Ala Arg Asn Leu Asp Trp Ile Asn Leu Met Ala Tyr  
35 195 200 205

Asp Phe Tyr Gly Pro Asn Trp Ser Pro Ser Gln Thr Asn Ser His Ala  
210 215 220

40 Gln Leu Phe Asp Pro Val Asn His Val Ser Gly Ser Asp Gly Ile Asn  
225 230 235 240

Ala Trp Ile Gln Ala Gly Val Pro Thr Lys Lys Leu Val Leu Gly Ile  
245 250 255

45 Pro Phe Tyr Gly Tyr Ala Trp Arg Leu Val Asn Ala Asn Ile His Gly  
260 265 270

Leu Arg Ala Pro Ala Ala Gly Lys Ser Asn Val Gly Ala Val Asp Asp  
50 275 280 285

Gly Ser Met Thr Tyr Asn Arg Ile Arg Asp Tyr Ile Val Glu Ser Arg  
290 295 300

45

Ala Thr Thr Val Tyr Asn Ala Thr Ile Val Gly Asp Tyr Cys Tyr Ser  
305 310 315 320

Gly Ser Asn Trp Ile Ser Tyr Asp Asp Thr Gln Thr Val Arg Asn Lys  
5 325 330 335

Val Asn Tyr Val Lys Gly Arg Gly Leu Leu Gly Tyr Phe Ala Trp His  
340 345 350

10 Val Ala Gly Asp Gln Asn Trp Gly Leu Ser Arg Thr Ala Ser Gln Thr  
355 360 365

Trp Gly Val Ser Phe Gln Glu Met Lys  
370 375

15 (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:  
20 (A) LENGTH: 17 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

30 AATACGACTC ACTATAG

17

(2) INFORMATION FOR SEQ ID NO: 10:

- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

45 TCTCCGGCAA CTAGTTTGCA C

21

(2) INFORMATION FOR SEQ ID NO: 11:

- 50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTCATAGTTC ACATATCTGT TGCC

24

(2) INFORMATION FOR SEQ ID NO: 12:

10

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TTTAAACTCG AAGTCACAAA TTAAA

25

25 (2) INFORMATION FOR SEQ ID NO: 13:

30

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TTTAAACTCG GATCCACAAA TTAAA

25

40 (2) INFORMATION FOR SEQ ID NO: 14:

45

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AATTGTCTAG AC

12

## CLAIMS

1. A plant protein isolated from other plant proteins, said plant protein having endo-chitinase activity, antifungal activity and a molecular weight of about 40 to 43 kDa as judged by Sodium Dodecyl Sulphate Polyacrylamide electrophoresis.
2. A plant protein isolated from other plant proteins according to claim 1, which is obtainable from leaves of TMV-induced tobacco plants.
3. An isolated protein according to claim 1, comprising an amino acid sequence as represented in SEQIDNO: 1 or 7, or a fragment thereof having chitinase activity.
4. A protein according to any one of the claims 1 to 3, wherein one or more amino acids is substituted, deleted, or added without abolishing chitinase activity.
5. An antifungal composition comprising as active ingredient a protein according to any one of the claims 1 to 4.
6. An antifungal composition according to claim 5, which further comprises  $\beta$ -1,3-glucanase and/or chitinase.
7. An antifungal composition according to claim 5, which comprises in addition to said protein a further protein acting synergistically with the said protein on fungi.
8. An isolated DNA sequence which comprises an open reading frame encoding a protein according to claim 1.
9. An isolated DNA sequence which comprises an open reading frame encoding the amino acid sequence of SEQIDNO: 1 or SEQIDNO: 7, and DNA sequences hybridising therewith under stringent conditions.
10. A plant expressible recombinant DNA sequence which comprises a DNA sequence encoding a protein according to claim 1, or a precursor of said

protein, said DNA sequence being physically linked to regulatory elements necessary for expression of said DNA sequence in plants in the form of said protein.

5 11. A plant expressible recombinant DNA sequence according to claim 10, wherein said DNA sequence encodes a protein precursor having the amino acid sequence represented by SEQIDNO: 1 or 7.

12. A plant expressible recombinant DNA sequence according to claim 10, 10 which comprises the DNA sequence as represented in SEQIDNO: 1 or 7.

13. A cloning vehicle or a vector containing a DNA sequence according to claim 10.

15 14. A bacterial strain containing a cloning vehicle or vector according to claim 13.

15. A bacterial strain of the genus *Agrobacterium* containing a vector according to claim 13.

20 16. A plant genome which has incorporated therein a plant expressible recombinant DNA sequence according to claim 10.

17. A plant cell which has a recombinant plant genome according to claim 16, 25 or a protoplast of said cell.

18. A plant or a material thereof, such as a bulb, flower, fruit, leaf, pollen, root or root culture, seed, stalk, tuber or microtuber, and the like, containing one or more cells according to claim 20.

30 19. A plant or material thereof, such as a bulb, flower, fruit, leaf, pollen, root or root culture, seed, stalk, tuber or a microtuber, and the like, wherein substantially all cells have a recombinant plant DNA genome according to claim 16.

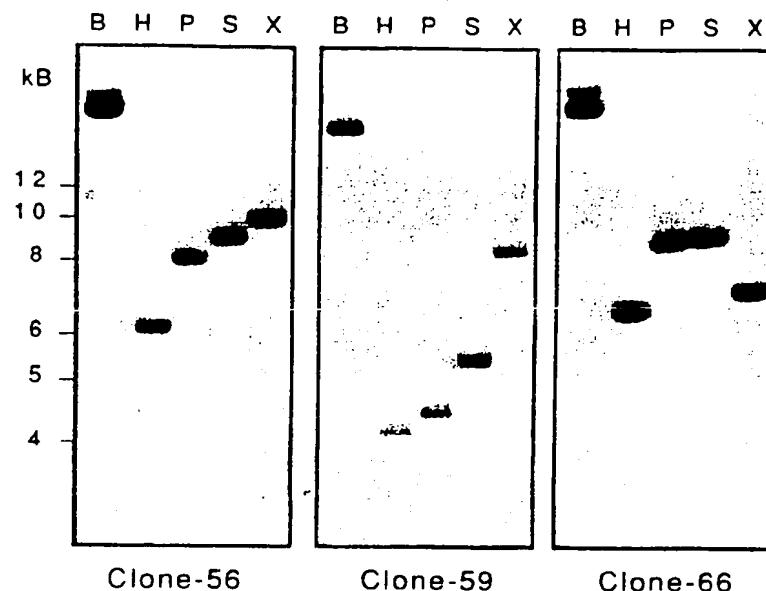
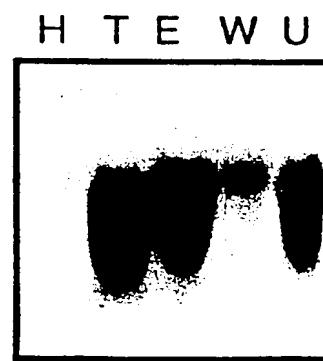
20. A plant, or material thereof, according to claim 19, which plant or material thereof is less susceptible to fungal infection.
21. A method for breeding a plant variety which has reduced susceptibility  
5 to fungi, characterized in that at least one of the parental lines has a recombinant DNA genome according to claim 16.
22. A method for inhibiting growth and/or germination of a fungus, by contacting the fungus, or causing the fungus to be contacted with an  
10 effective amount of a chitinase according to claim 1.

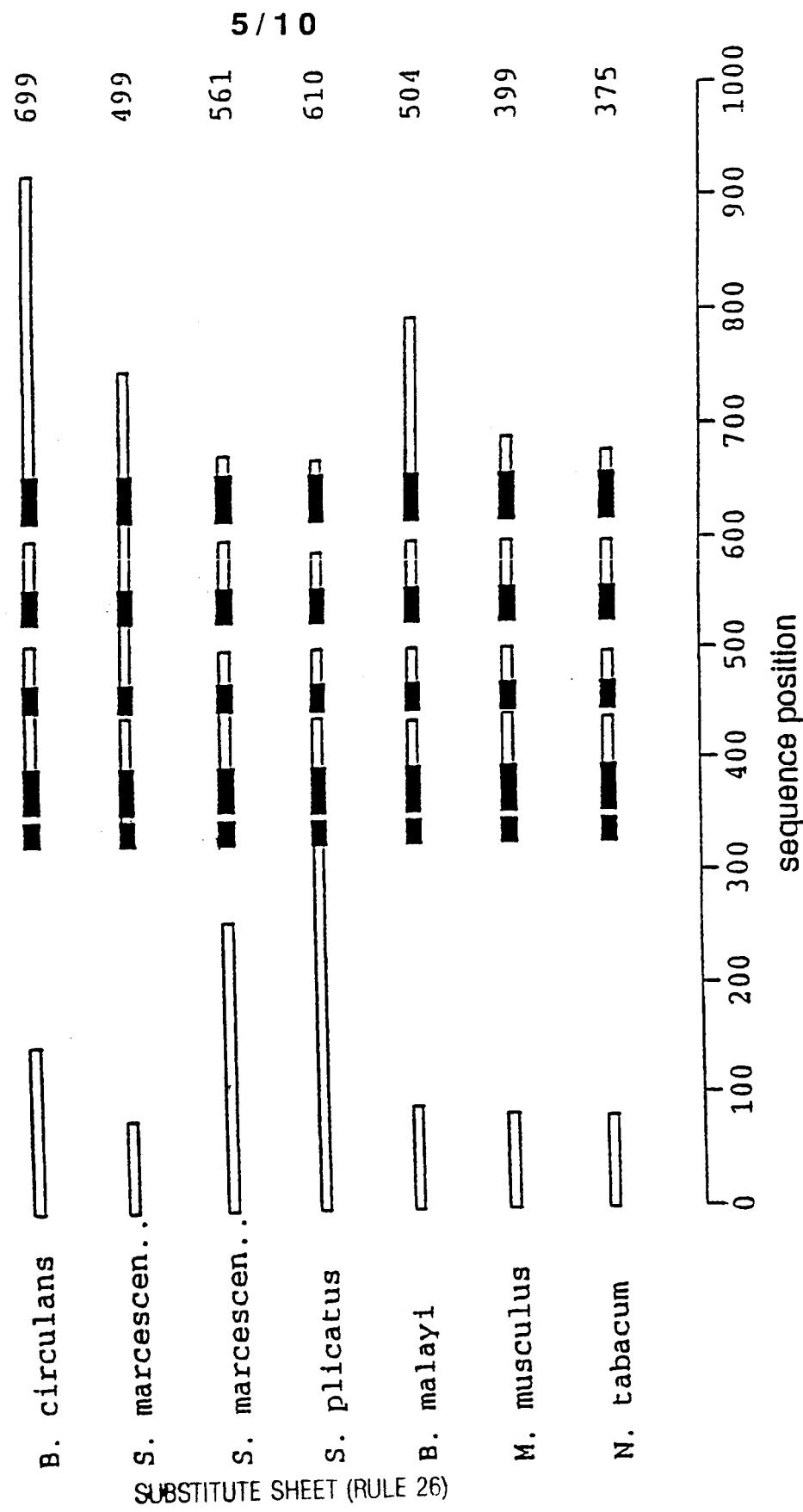


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**Figure 1c**

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**Figure 2****Figure 3**

**Figure 4**

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(a)

(b)

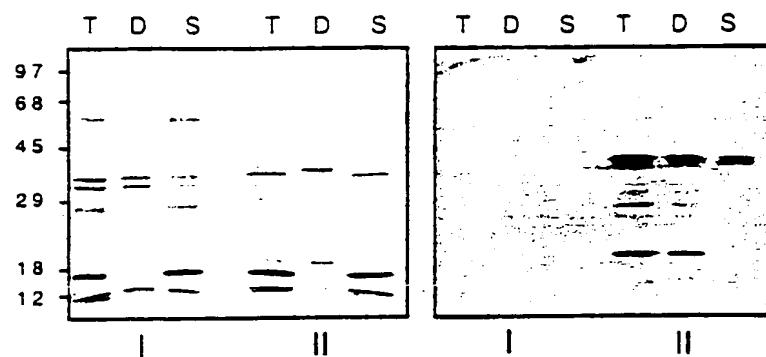


Figure 5

Mr A B C D

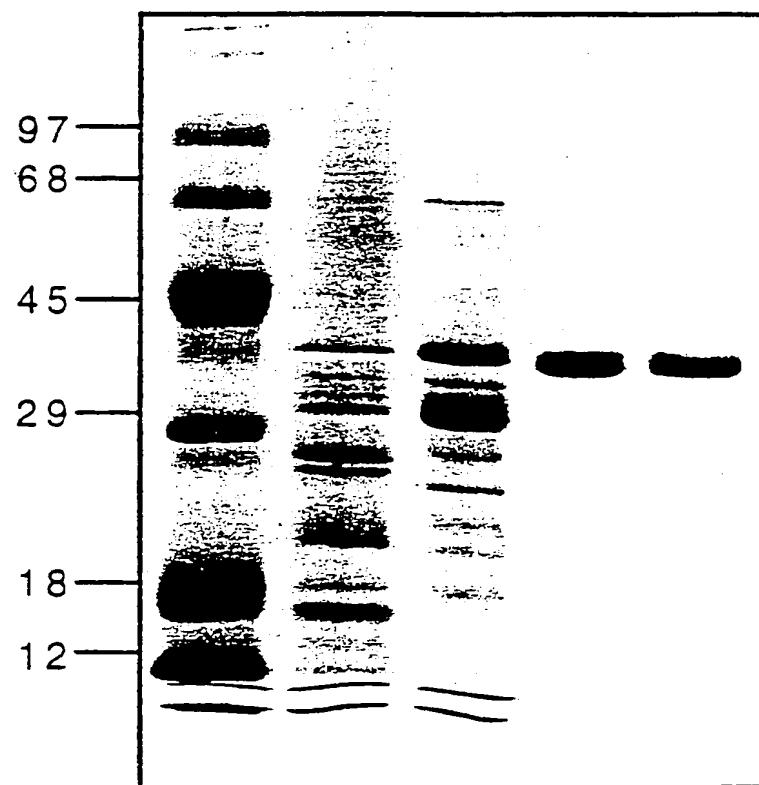


Figure 6

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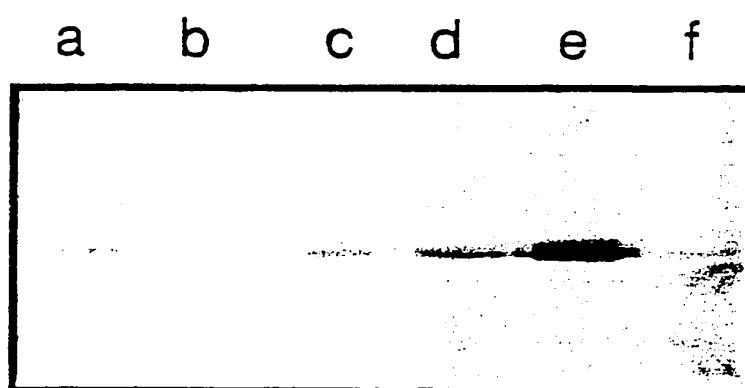


Figure 7

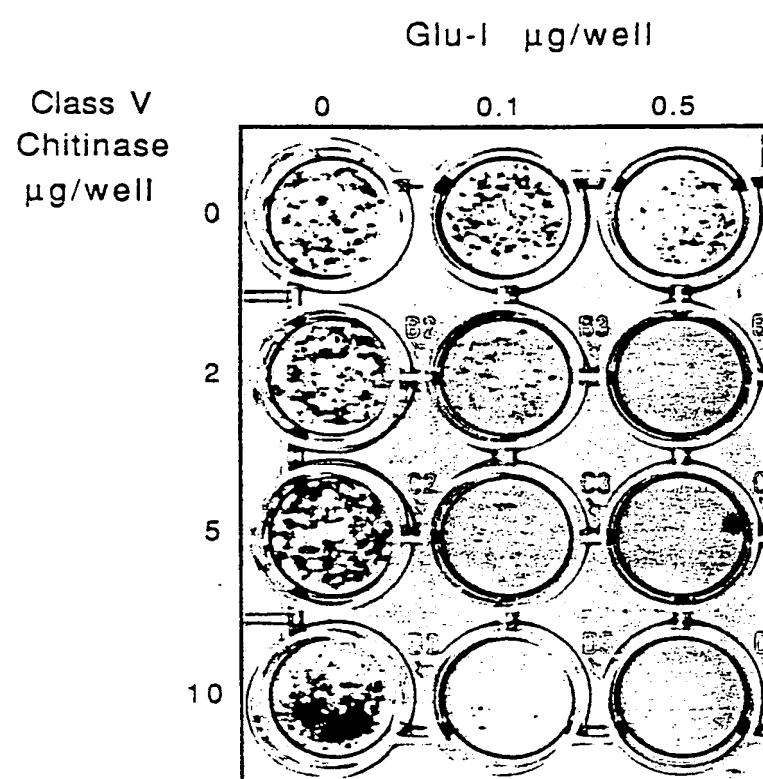


Figure 8

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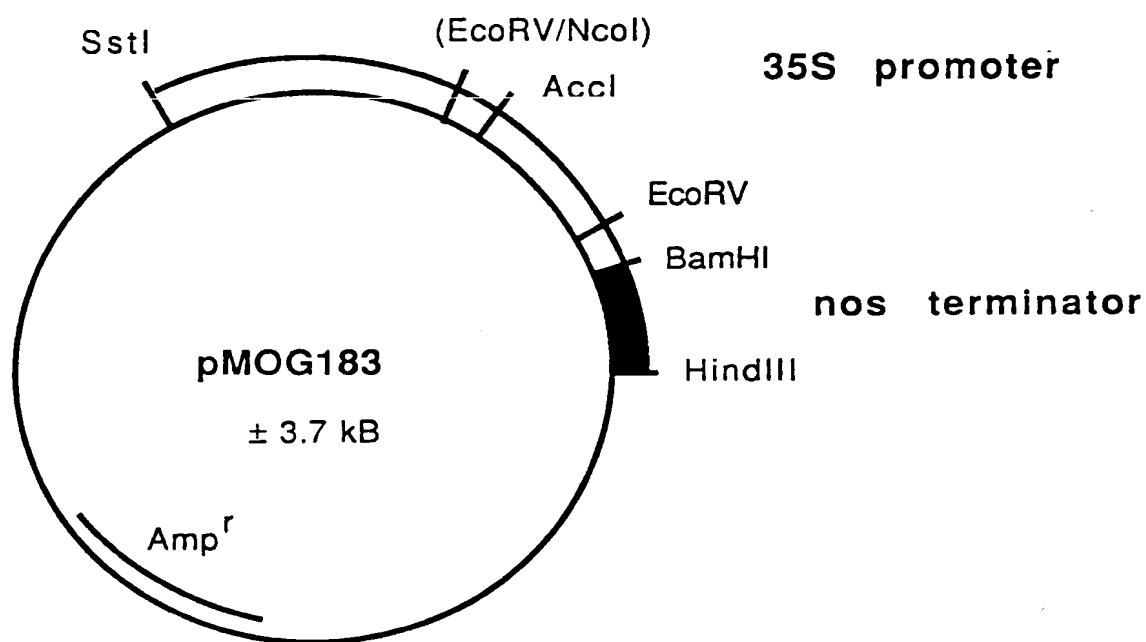


Figure 9

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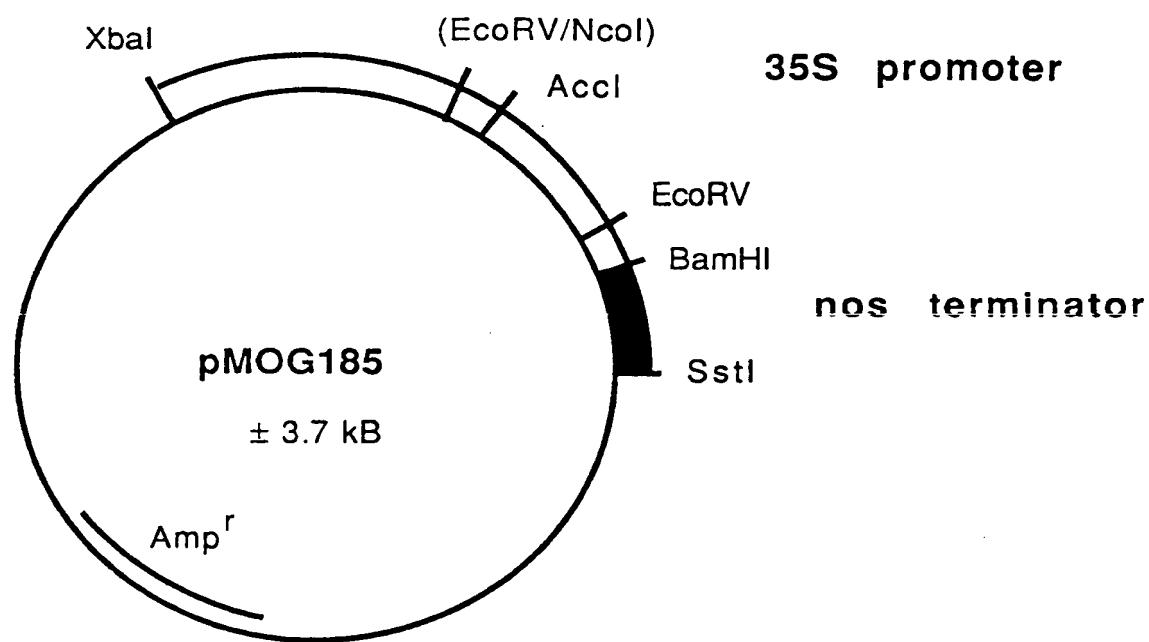
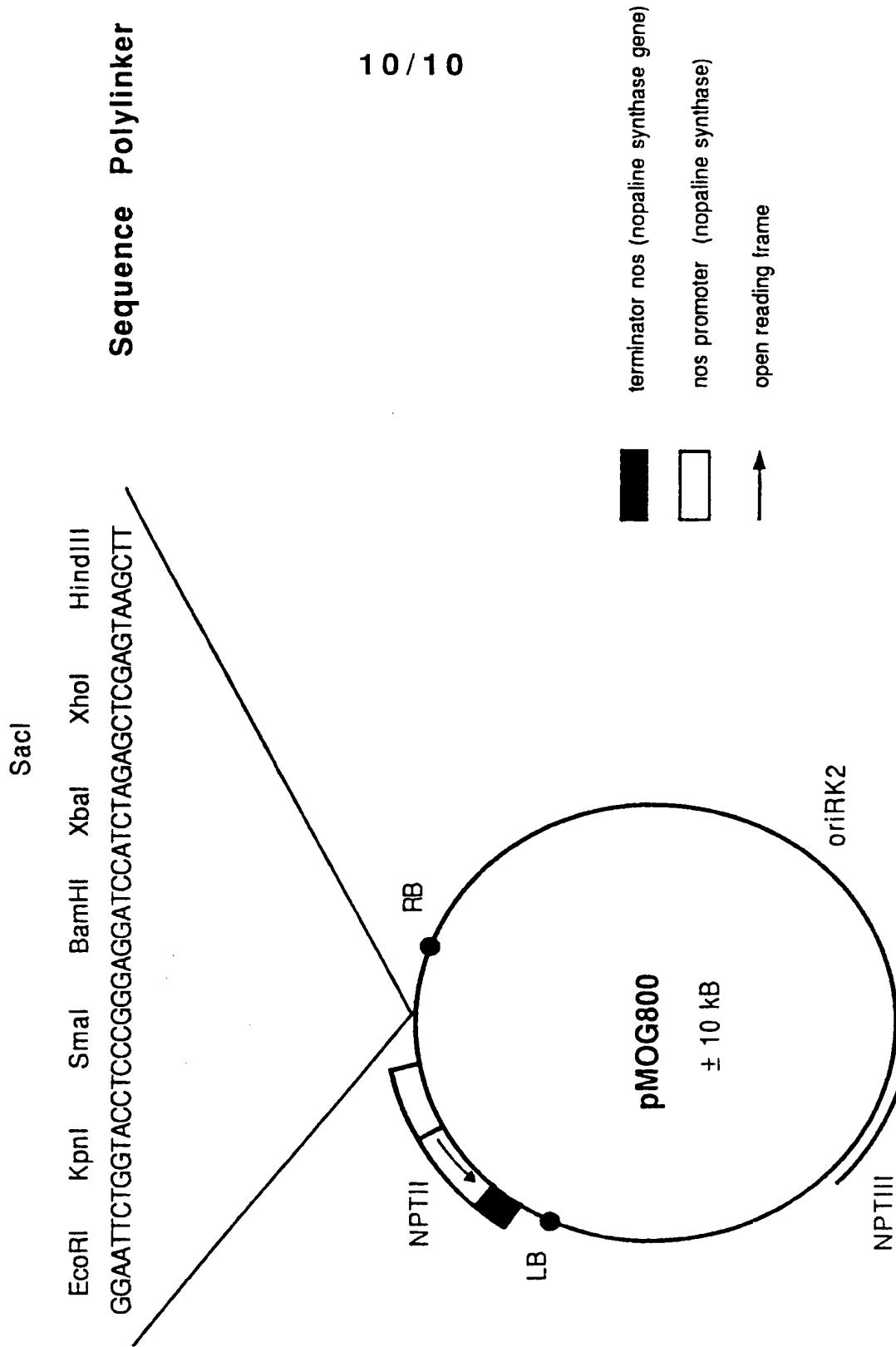
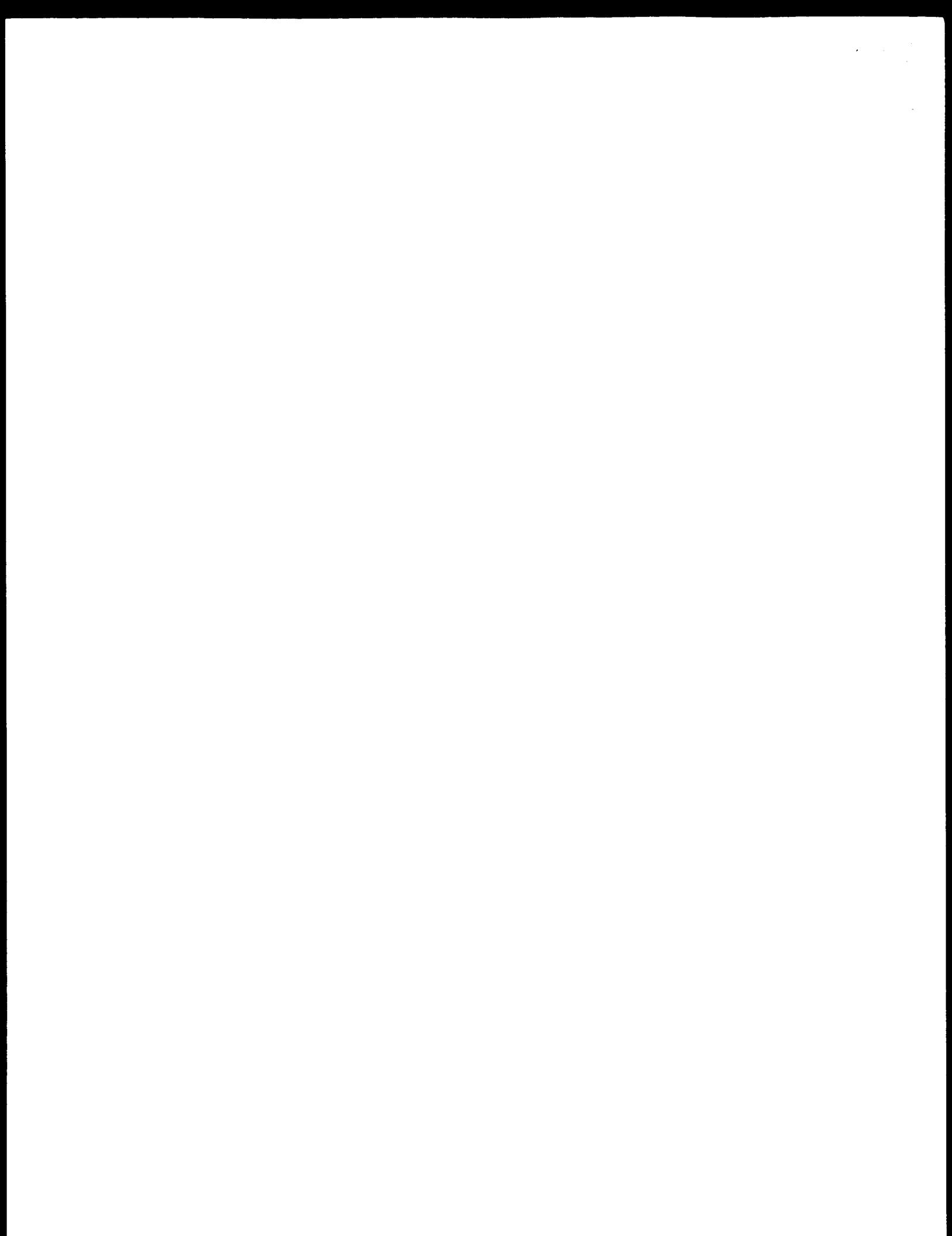


Figure 10

**Figure 11**

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12N 15/56, 15/82, C07K 14/415, C12N 9/24, 5/10, 1/21, A01H 5/00, A01N 65/00</b>		A3	(11) International Publication Number: <b>WO 95/05467</b> (43) International Publication Date: 23 February 1995 (23.02.95)
(21) International Application Number: <b>PCT/EP94/02761</b>		MR Leiden (NL). SELA-BUURLAGE, Marianne, Beatrix [NL/NL]; Edisonstraat 59, NL-3817 VP Amersfoort (NL).	
(22) International Filing Date: 17 August 1994 (17.08.94)		(74) Common Representative: MOGEN INTERNATIONAL N.V.; Einsteinweg 97, NL-2333 CB Leiden (NL).	
(30) Priority Data: 93202425.0 17 August 1993 (17.08.93) EP (34) Countries for which the regional or international application was filed: NL et al.		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).	
(71) Applicants (for all designated States except US): MOGEN INTERNATIONAL N.V. [NL/NL]; Einsteinweg 97, NL-2333 CB Leiden (NL). RIJKSUNIVERSITEIT TE LEIDEN [NL/NL]; Stationsweg 46, NL-2312 AV Leiden (NL).		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(72) Inventors; and (75) Inventors/Applicants (for US only): APOTHEKER-DE GROOT, Marion [NL/NL]; Engelandlaan 1248, NL-2034 GM Haarlem (NL). BOL, John, Ferdinand [NL/NL]; Lange Voort 34, NL-2341 KA Oegstgeest (NL). CORNELISSEN, Bernardus, Johannes, Clemens [NL/NL]; Herenweg 29, NL-2361 EB Warmond (NL). LINTHORST, Hubertus, Josephus, Maria [NL/NL]; Ank van der Moerstraat 70, NL-2331 HS Leiden (NL). MELCHERS, Leo, Sjoerd [NL/NL]; Wilhelmina Bladergroenweg 45, NL-2331 BZ Leiden (NL). PONSTEIN, Anne, Silene [NL/NL]; Meerforel 5, NL-2318		(88) Date of publication of the international search report: 23 March 1995 (23.03.95)	

(54) Title: CHITINASE, DNA CODING THEREFOR AND PLANTS CONTAINING SAME

## (57) Abstract

The present invention provides a novel class of plant chitinases, which have endo-chitinase activity, antifungal activity and a molecular weight of about 40 to 43 kDa as judged by Sodium Dodecyl Sulphate Polyacrylamide electrophoreses. The invention further provides antifungal compositions comprising as active ingredient a chitinase according to the invention. According to a different aspect of the invention an isolated DNA sequence is provided which comprises an open reading frame encoding a novel chitinase. The invention also encompasses a plant expressible recombinant DNA sequence which comprises a DNA sequence encoding a chitinase according to the invention, as well as plant cells and plants which have such recombinant DNA incorporated into their genome.

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## INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/02761

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/56	C12N15/82	C07K14/415	C12N9/24	C12N5/10
C12N1/21	A01H5/00	A01N65/00		

According to International Patent Classification (IPC) or to both national classification and IPC

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NATO ASI SERIES, SERIES H: CELL BIOLOGY, SIGNAL MOLECULES IN PLANTS AND PLANT-MICROBE INTERACTIONS, EDITED BY BEN J.J. LUGTENBERG, vol.36, 1989 pages 161 - 168</p> <p>FRITIG, B., ET AL. 'Virus-induced glycanhydrolases and effects of oligosaccharide signals on plant-virus interactions' see page 163; figure 1</p> <p>----</p> <p style="text-align: center;">-/-</p>	1-5

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Date of the actual completion of the international search

19 January 1995

Date of mailing of the international search report

06-02-1995

## Name and mailing address of the ISA

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE PLANT JOURNAL, vol.5, no.4, 1994 pages 469 - 480 MELCHERS, L.S., ET AL. 'A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity' see the whole document ---	1-5,8,9, 22
A	EMBO JOURNAL, vol.5, no.9, 1986, EYNSHAM, OXFORD GB pages 2057 - 2061 HOOFT VAN HUIJSDUIJNEN, R.A.M., ET AL. 'cDNA cloning of six mRNAs induced by TMV infection of tobacco and a characterization of their translation products' see the whole document ---	1-22
A	PLANT GENE RESEARCH, TEMPORAL AND SPATIAL REGULATION OF PLANT GENES; VERMA, D., ET AL. EDS, 1988 pages 201 - 221 BOL, J.F., ET AL. 'Structure and expression of plant genes encoding pathogenesis-related proteins' see page 210 - page 211; table 2 ---	1-22
A	BIOCHIMIE, vol.75, no.8, 1993 pages 687 - 706 STINTZI, A., ET AL. 'Plant 'pathogenesis-related' proteins and their role in defense against pathogens' see table VII ---	1-22
A	EP,A,0 440 304 (MOGEN) 7 August 1991 see the whole document ---	1-22
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